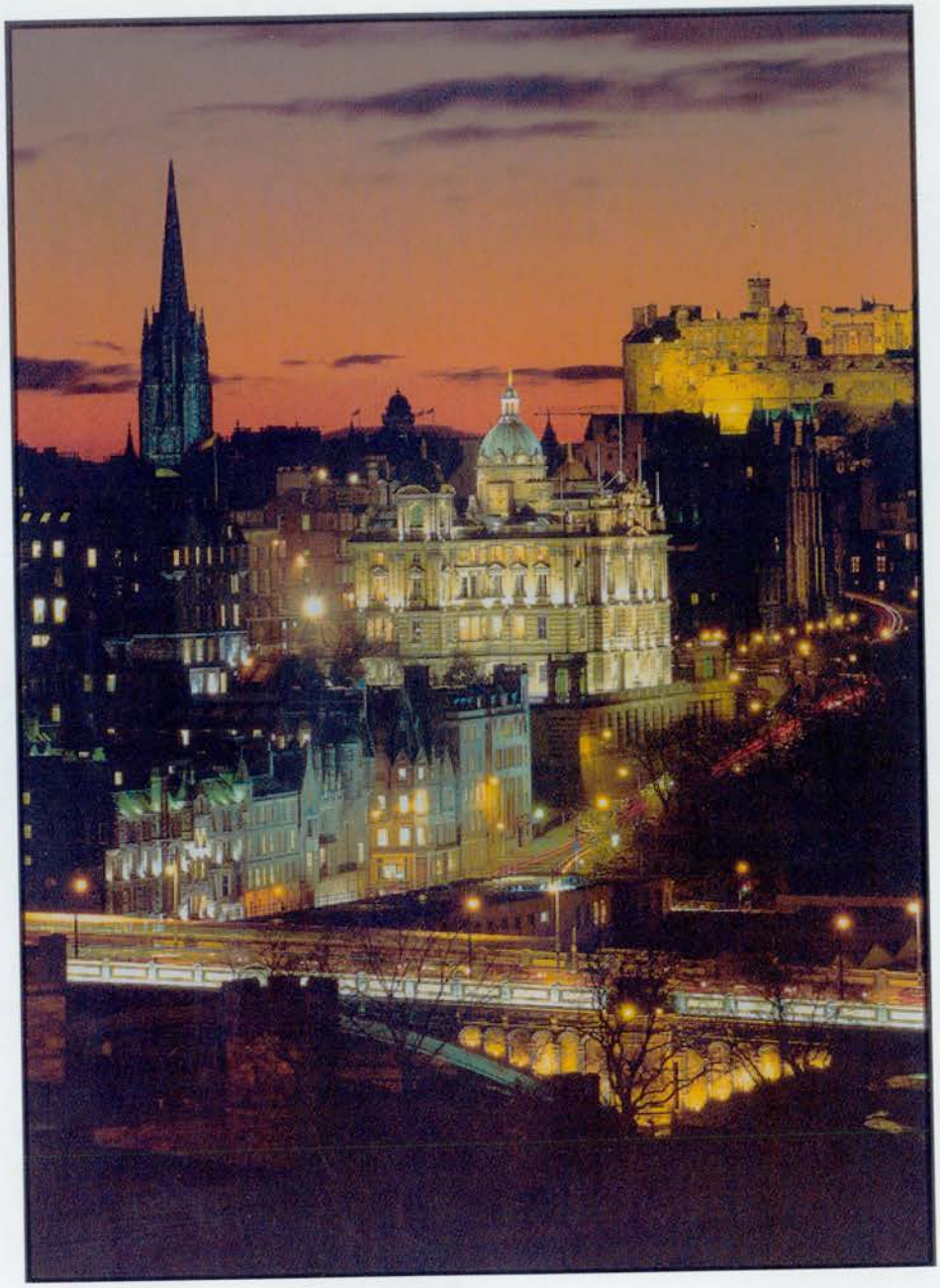
Central Mechanisms of Reduced Neuroendocrine Stress Responses in Pregnancy

Paula J Brunton

PhD
The University of Edinburgh
2002



Edinburgh

Declaration

The experiments described in this thesis were performed by the author in the Laboratory of Neuroendocrinology within the Division of Biomedical and Clinical Laboratory Sciences at the University of Edinburgh under the supervision of Professor John A Russell. It is clearly stated in the text where experiments involved collaboration with others. This thesis was composed solely by the author and no part has been previously presented for a degree, diploma or other qualification at this or any other university.

Paula J Brunton

January 2002

for my parents

Acknowledgements

Firstly I would like to express my sincere thanks and gratitude to my supervisor Professor John Russell for his support, enthusiasm for research, encouraging pep talks and most importantly his sense of humour. I have benefited immensely from his expertise and guidance throughout my research and during the preparation of this thesis. I would also like to thank John for giving me the opportunity to present my data at conferences both here and abroad.

I would like to extend my thanks to the Faculty of Medicine Awards Committee for awarding me with the Goodsir Memorial Scholarship which provided me with financial support during my PhD and the BBSRC for funding the research.

I have many colleagues to thank for their assistance in the lab, especially with teaching me new techniques. Dr. Alison Douglas (my surrogate second supervisor!) for teaching me and assisting with surgical techniques. Dr. Philip Bull for assisting me in surgery and coaching me through the oxytocin radioimmunoassay. Prof. Joe McCabe who gave his time freely to help me in the beginning with *in situ* hybridisation and Dr. Gordon Blackburn-Munro for all his technical advice.

My appreciation is also extended to Prof. Gareth Leng, Dr. Mike Shipston and Dr. Alison Douglas for their valuable discussions of my data.

I must also thank Dr. Inga Neumann for giving me the opportunity to work with her at the Max Planck Institute for Psychiatry in Munich and Miss Simone Kröemer, Dr. Alexandra Wigger and Prof. Rainer Landgraf for their hospitality while I was there.

To the staff in the medical faculty animal house, my thanks for their efforts in provision and maintenance of the animals used in these experiments, especially Carol, Duncan, Graeme, Pat, Don and Vince.

I have made many friends during my time in the Laboratory of Neuroendocrinology and would like to thank them. For all the laughs, cakes, visits to the pub and more recently for putting up with my moaning during the thesis writing, Dr. Simone Meddle, Dr. Alex Johnston, the girls in A218, Miss Céline Caquineau, Mrs Valerie Bishop, Dr. Nancy Sabatier and in the early days Drs. Colin Brown and Gordon Blackburn-Munro.

Finally I would like to express my deepest thanks to my parents, Jane and Raymond and to my partner, Mustapha for all their love, support, encouragement and patience throughout my long career as a student. No more degrees, I promise!

Abstract

The hypothalamo-pituitary-adrenal (HPA) axis plays a vital role in restoring homeostasis following stress; responsiveness of the axis can change however. The adrenocorticotropic hormone (ACTH) and corticosterone secretory responses to emotional and physical stressors are reduced in late pregnancy. Here, experiments were designed to investigate changes in the brain mechanisms underlying these reduced responses, with particular focus on adaptations at the level of the hypothalamus. The responsiveness of the HPA axis to the emotional stressors, restraint and maternal aggression were markedly reduced in late pregnant (day 21) rats. This was at least partly a result of reduced activation of the parvocellular paraventricular nucleus (pPVN) corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) neurones, as revealed by quantitative *in situ* hybridisation for NGFI-B mRNA and AVP hnRNA expression. To seek changes in responses to immune challenge, lipopolysaccharide (LPS) and interleukin-1β (IL-1β) were given acutely. Blood sampling from conscious rats revealed that the ACTH and corticosterone secretory responses to peripheral immune challenge are strongly attenuated in late pregnant rats. This was found to be a consequence of the CRH neurones being stimulated less by the stressor in pregnancy, resulting in reduced drive to the anterior pituitary by CRH. IL-1β has been shown to act via a brainstem pathway from the nucleus of the tractus solitarus (NTS) to the PVN. IL-1β was found to activate Fos expression in NTS neurones similarly in virgin and pregnant rats, indicating that signalling between the NTS and the PVN neurones is interrupted in pregnancy, resulting in reduced activation of the HPA axis. Endogenous opioids are involved in this attenuation of HPA axis responses to IL-1β in pregnancy, as removing opioid inhibition by pre-treatment with the opioid receptor antagonist, naloxone, largely reinstated the ACTH response to IL-1β in the pregnant rats, and resulted in CRH mRNA responses that were not different between virgin and pregnant rats. Oxytocin is also a "stress hormone" in rats. Oxytocin secretory responses to systemic IL-1β were attenuated in late pregnant rats, which was shown to be also a consequence of strong opioid restraint. To test if CRH neurones were also non-responsive in pregnancy to excitatory stimuli concerned with regulating metabolism and arousal, the effects of orexin-A on HPA activity was measured. Intracerebroventricular administration of orexin-A evoked a response at all levels of the HPA axis in virgin rats, but failed to activate the HPA axis in late pregnant rats, indicating a "*global reduction*" in the responsiveness of the CRH neurones to stimuli in late pregnancy. A possible role for enhanced slow and rapid corticosterone negative feedback mechanisms was investigated. In pregnancy, slow glucocorticoid feedback appears to be enhanced so that the CRH and AVP neurones in the PVN are more sensitive to removal of this feedback signal. In contrast, sensitivity to rapid corticosterone feedback in response to stress is reduced, and is unlikely to underlie the attenuated HPA axis responses to stressors in pregnant rats. A comparative study demonstrated that mice also display attenuated HPA axis responses to stress in late pregnancy. Thus changes in central drive to the PVN neurones and their responsiveness to excitatory inputs seems to be the most important factor in the reduced responsiveness of the HPA axis in late pregnancy, though enhanced slow glucocorticoid negative feedback may contribute. The significance of the suppression of CRH/AVP pPVN neurone responsiveness, and hence reduced HPA responses in rats and mice following exposure to stress in pregnancy is that it will limit exposure of the fetuses to excessive levels of glucocorticoids.

Contents

List of Figures

List of Tables

List of Abbreviations

α-MSH	alpha-melanocyte stimulating hormone
A1	catecholamine cell group
A2	catecholamine cell group
A5	catecholamine cell group
ac	anterior commissure
aCSF	artificial cerebrospinal fluid
ACTH	adrenocorticotropic hormone
ADX	adrenalectomy
AG	aminoglutethimide
AGRP	agouti related peptide
ANOVA	analysis of variance
AP	area postrema
A. Pit	anterior pituitary gland
AVP	arginine vasopressin
AVT	arginine vasotocin
β-LPH	beta-lipotropin
β-MSH	beta-melanocyte stimulating hormone
BBB	blood-brain-barrier
Bmax	maximum binding
BNST	bed nucleus of the stria terminalis
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CART	cocaine & amphetamine regulated transcript
CBG	corticosterone binding globulin
CBX	carbenoxolone
cc	corpus callosum
CCK	cholecystokinin
cDNA	complementary deoxyribonucleic acid
CeA	central nucleus of the amygdala
CG	central gray
CLIP	corticotropin-like intermediate lobe peptide
CNS	central nervous system
cNTS	commissural NTS
COX	cyclo-oxygenase
cpm	counts per minute
CREB	cAMP response element binding protein
CRF	corticotropin-releasing factor
CRH	corticotropin-releasing hormone
CRH-BP	CRH binding protein
CRH-R	CRH receptor
CRH-R_1	CRH receptor type 1
CRH-R_2	CRH receptor type 2
CVO	circumventricular organ

DAB	diaminobenzidine
ddH_2O	double distilled water
dH_2O	single distilled water
DEPC	diethylpyrocarbonate
DG	dentate gyrus
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DRN	dorsal raphe nucleus
DTT	dithiothreitol
DVC	dorsal vagal complex
EDTA	ethylenediamineteraacetic acid
EP_{1-4}	prostaglandin receptor subtypes
EPM	elevated plus maze
FSH	follicle stimulating hormone
GA	glycyrrhetinic acid
GABA	gamma-aminobutyric acid
$GABA_A$	gamma-aminobutyric acid receptor type A
γ-MSH	gamma- melanocyte stimulating hormone
GH	growth hormone
GR(s)	glucocorticoid receptor(s)
GRE	glucocorticoid response element
h	hour(s)
11β-HSD	11-beta-hydroxy-steroid dehydrogenase
HIP	hippocampus
hnRNA	heteronuclear ribonucleic acid
HNS	hypothalamo-neurohypophysial system
HPA	hypothalamo-pituitary-adrenal
IEG(s)	immediate early gene(s)
i.c.v.	intracerebroventricular
IL-1α	interleukin-1-alpha
IL-1β	interleukin-1-beta
IL-6	interleukin-6
i.p.	intraperitoneal
IP	inositol phosphate
ir	immunoreactive
ISH	*in situ* hybridisation
i.v.	intravenous
kb	kilobase
kDa	kilo-Dalton

LC	locus coeruleus
LDT	laterodorsal tegmental nucleus
LH	luteinising hormone
LHA	lateral hypothalamic area
LPB	LPS-binding protein
LPS	lipopolysaccharide
LV	lateral ventricle
4-MA	5α reductase inhibitor
MD	maternal defence
ME	median eminence
MET	metyrapone
mfb	medial forebrain bundle
mgPVN	magnocellular division of PVN
MID THAL	midline thalamic nuclei
MIF	melanocyte-stimulating hormone inhibiting factor
min	minute(s)
mPOA	medial preoptic area
MR(s)	mineralocorticoid receptor(s)
MRN	median raphe nucleus
mRNA	messenger ribonucleic acid
MVN	median vestibular nucleus
NA	noradrenaline
NBRE	NGFI-B response element
NGFI-B	nerve growth factor induced gene –B
NLX	naloxone
NPY	neuropeptide Y
NSB	non-specific binding
NTS	nucleus of the solitary tract or nucleus of the tractus solitarius
OC	optic chiasm
oCRH	ovine CRH
ORX-A	orexin-A
OX_1	orexin receptor type 1
OX_2	orexin receptor type 2
OVLT	organum vasculosum of the lamina terminalis
OXT	oxytocin
PB	parabrachial nucleus
PBS	phosphate buffered saline
pCREB	phospho-CREB
pDYN	prodynorphin
pENK	proenkephalin
PFA	prefrontal area
PG(s)	prostaglandin(s)
PGE_1	PG subtypes
PGE_2	PG subtypes
$PGF_{2\alpha}$	PG subtypes

phADX		pharmacological ADX
PLC		phospholipase C
POMC		proopiomelanocortin
POR		perioculomotor raphe region
preNS		prenatal stress
PRL		prolactin
PP		posterior pituitary gland
pPVN		parvocellular region of the paraventricular nucleus
	ap	anterior parvocellular regior
	mp	medial parvocellular
	lp	lateral parvocellular
	dp	dorsal parvocellular
	pv	periventricular parvocellulai
PVN		paraventricular nucleus
PVT		paraventricular thalamic nucleus
REM		rapid eye movement
RN		raphe nucleus
rPL		rat placental lactogen
s.c.		subcutaneous
SCN		suprachiasmatic nucleus
SCO		subcommissural organ
SD		standard deviation
SEM		standard error of the mean
SEPT		septum
SFO		subfornical organ
SI		substantia innominata
st		stria terminalis
SON		supraoptic nucleus
SSC		standard sodium citrate buffer
TC		total counts
TdT		terminal deoxynucleotidyl transferase
TEA		triethanolamine
TNF-α		tumour necrosis factor alpha
TRH		thyrotrophin-releasing hormone
TSH		thyroid stimulating hormone
3V		third ventricle
V_{1a}		vasopressin receptor subtypes
V_{1b}		
V_2		
VEH		vehicle
VLM		ventrolateral medulla
VMH		ventromedial hypothalamus
VSA		ventral septal area

subdivisions of pPVN

CHAPTER 1

General Introduction

1.1. Introduction

The successful existence of an organism is dependent upon its ability to mount an appropriate response when faced with a physiological challenge such as stress. Stress is a widely used term, with meanings in many disciplines. In the medical field, Hans Selye (Selye H., 1936) first defined stress as the generalised effort of the organism to adapt to a critical situation and termed this response "general adaptation syndrome".

Today the popular perception of stress recognised by most civilisations around the world is that of social and/or work pressures that are often associated with increased frequency of illnesses. However, stress is not all bad. Responding to stress is a vital defence mechanism, without which an organism's life may be threatened. Exposure to adverse events results in a series of orchestrated responses often referred to as 'stress responses'. These responses act to preserve homeostasis and involve complex behavioural, autonomic and endocrine adaptations, which are all aimed at enhancing the chances of survival. Problems occur when an organism fails to terminate a response to an acute stress and can result in chronic stress syndrome, characterised by elevated secretion of glucocorticoids with consequent immunosuppression and vulnerability to disease (Jessop, 1999). Thus, health relies on an organism's ability to respond to a stressful situation but also on its ability to switch off the stress response appropriately, when the stress no longer poses a threat.

1.2. Stress and 'Fight-Flight' Responses

The hypothalamo-pituitary-adrenal (HPA) axis and the sympathetic adrenomedullary systems are the key players in expressing stress responses. Activation of the HPA axis as a result of stress triggers a neuroendocrine cascade of events, the final outcome of which is increased production and secretion of glucocorticoids (e.g. corticosterone in the rat) from the adrenal cortex. The principal action of glucocorticoids is to promote mobilisation of glucose and fat stores and stimulate gluconeogenesis (Jeanrenaud & Rohner-Jeanrenaud, 2001). Glucocorticoids also play an important role in restraining the effectors of the stress response. During stress, the sympathetic adrenomedullary system is also activated, leading to release

of catecholamines (for review see (McCarty, 1994) e.g. adrenaline and noradrenaline from the adrenal medulla. In 1930 Walter Cannon (Cannon, 1930) described actions of the "involuntary" (autonomic) nervous system in response to changes in the internal environment. The autonomic nervous system plays its part by actions upon the cardiovascular and respiratory systems, smooth muscles and glands.

Glucocorticoids and catecholamines act together to mobilise energy stores and redistribute resources. They raise blood pressure and cardiac output and enhance the delivery of substrates to tissues that are critical to the immediate defence of the organism, thus enabling the organism to cope with emergency situations and facilitate "fight or flight" responses. The function of these adaptations is to preserve homeostasis and defend the stability of the internal environment.

1.3. The Hypothalamo-Pituitary-Adrenal Axis

The major endocrine response to stress occurs via activation of the hypothalamo-pituitary-adrenal (HPA) axis (see figure 1.1.), which functions to restore deviations from homeostasis and employs complex negative and positive feedback mechanisms (Campeau *et al*, 1998). Stress activates neurosecretory neurones in the parvocellular region of the hypothalamic paraventricular nucleus (pPVN). These neurones synthesise two neuropeptides: corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP). Upon activation of these neurones CRH and AVP is released from nerve terminals at the external zone of the median eminence into the hypophysial portal blood system (see figure 1.2.). CRH and AVP act synergistically at the level of the anterior pituitary to evoke the release of adrenocorticotropic hormone (ACTH). Specialised cells termed corticotrophs secrete ACTH, which in turn stimulates secretion of glucocorticoids (corticosterone in rodents or cortisol in humans) from the adrenal cortex. Glucocorticoids restrain their own release by providing feedback inhibition at several levels including the pituitary, hypothalamus and the hippocampus (see section 1.14.4.).

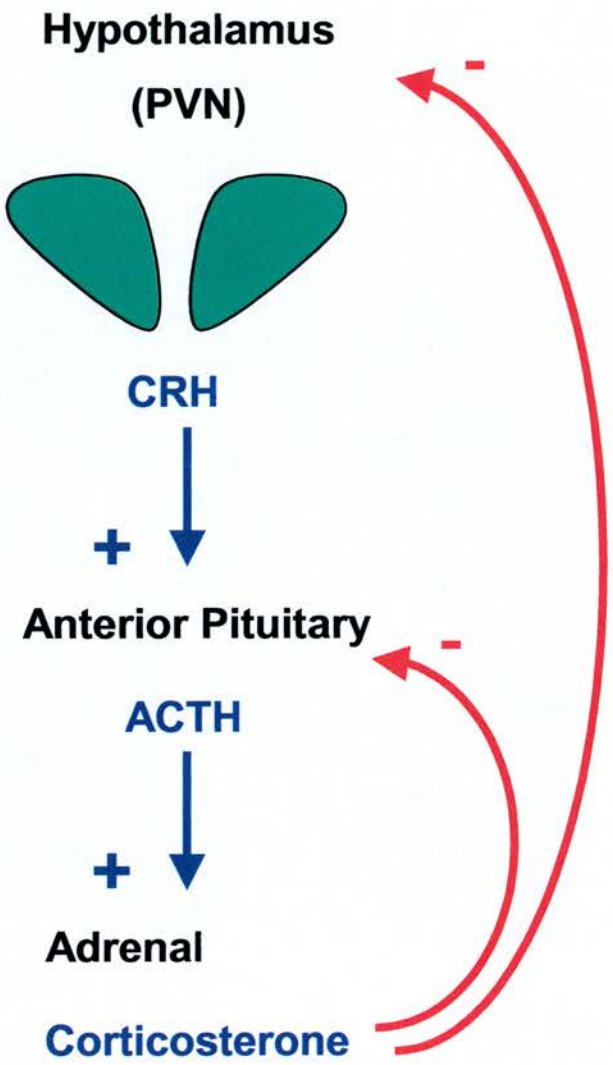


Figure 1.1. The Hypothalamo-Pituitary-Adrenal (HPA) Axis

Neurones in the parvocellular division of the paraventricular nucleus (pPVN) in the hypothalamus secrete corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) in response to stress. CRH stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland, which in turn triggers the release of corticosterone from the adrenal cortex. Corticosterone has a negative feedback effect at the level of both the anterior pituitary and the hypothalamus to restrain the HPA axis. Blue arrows indicate stimulation; red arrows indicate inhibition.

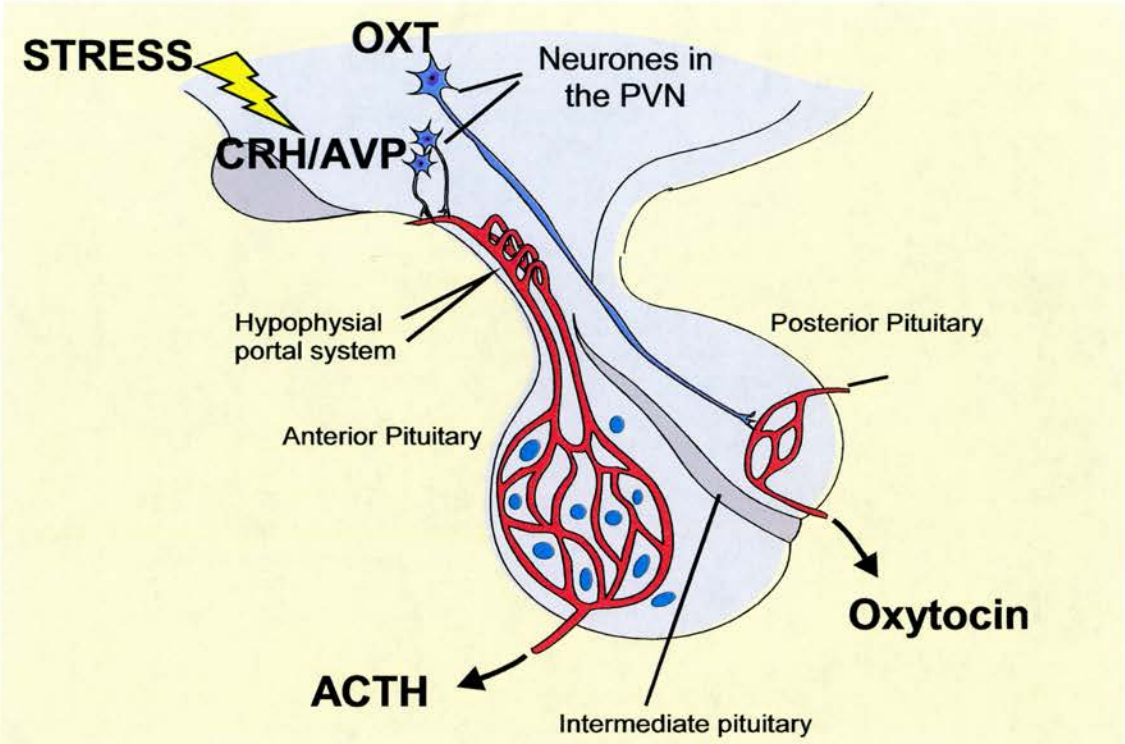


Figure 1.2. Hypothalamo-Pituitary Interactions

Stress activates CRH/AVP neurones in the parvocellular division of the paraventricular nucleus (pPVN). CRH/AVP neurones terminate at the median eminence, where they release their respective peptides into the hypophysial portal blood system. This portal system connects the hypothalamus and the pituitary. On binding with receptors on anterior pituitary corticotrophs, CRH and AVP stimulate release of ACTH. The diagram also shows oxytocin neurones which project directly from the PVN to the posterior pituitary. Oxytocin's role in stress is discussed in section 1.11. Abbreviations: CRH, corticotropin-releasing hormone; AVP, arginine vasopressin; ACTH, adrenocorticotropic hormone; OXT, oxytocin.

1.4. The Paraventricular Nucleus

The hypothalamus is situated at the base of the forebrain below the thalamus, and is divided in two by the third ventricle (see figure 1.3). Attached to the hypothalamus by the hypophysial stalk is the pituitary gland. The hypothalamus plays a critical role in the coordination of autonomic, endocrine and behavioural responses that maintain homeostasis (Morgane & Panksepp, 1980). In mammals the hypothalamus is involved in regulation of body temperature (Kanosue *et al*, 1998), the cardiovascular system (Ganten *et al*, 1985), body fluid homeostasis (Denton *et al*, 1996) and feeding (Lawrence *et al*, 1999) , as well as sexual and maternal behaviours (Naruse, 1997; Pfaus, 1999); all of which aid survival of the species. The neural mechanisms responsible for these adaptive responses are not completely understood and the neuroanatomical circuitry involved has been in the past difficult to study since the hypothalamus gives rise to and is traversed by complex fibre systems. Technological advancements in the methods used to study neuroanatomy have considerably benefited those researching the hypothalamus (for review see (Jones & Hatman, 1978)). For example, the development of fluorescent labelled markers for anterograde and retrograde labelling have provided information about neuronal axonal projections to and from the hypothalamus, and immunocytochemical techniques provide information about the identity of these cells.(Lawrence *et al* , 1999)

The primary hypothalamic region involved in neuroendocrine responses to stress is the paraventricular nucleus (PVN). The PVN is found on either side of the third ventricle (see figure 1.4.) and is composed of distinct magnocellular (large cell) and parvocellular (small cell) divisions (Gurdjian, 1927; Kreig, 1932). These two major areas of the PVN can be further divided into a total of eight distinct subdivisions based on cell structure, cell connections and cell type (Swanson & Kuypers, 1980), three of which are magnocellular and five of which are parvocellular. The three subdivisions of the magnocellular PVN are the (i) anterior magnocellular (amPVN), (ii) medial magnocellular (mmPVN) and (iii) posterior magnocellular (pmPVN) areas (Swanson & Sawchenko, 1980; Swanson & Sawchenko, 1983). Cells located in

these regions project primarily to the posterior lobe of the pituitary gland (Sherlock *et al*, 1975). Cells in the parvocellular division of the PVN (pPVN) generally project to the external lamina of the median eminence (Swanson *et al*, 1980; Wiegand & Price, 1980b) where they influence anterior pituitary function and to the brainstem and spinal cord (Hosoya & Matsushita M., 1979; Swanson & Kuypers, 1980). As mentioned above the parvocellular division can be divided into five distinct parts, these are: anterior, (ap); medial, (mp); lateral, (lp); dorsal, (dp) and periventricular, (pv) (Swanson & Sawchenko, 1980; Swanson & Sawchenko, 1983).

The PVN of the hypothalamus is the site of CRH/AVP producing cells and thus plays a crucial role for central control of the HPA axis. Lesions to this region markedly reduce portal blood CRH levels and stress induced ACTH and corticosterone secretion (Antoni, 1986).

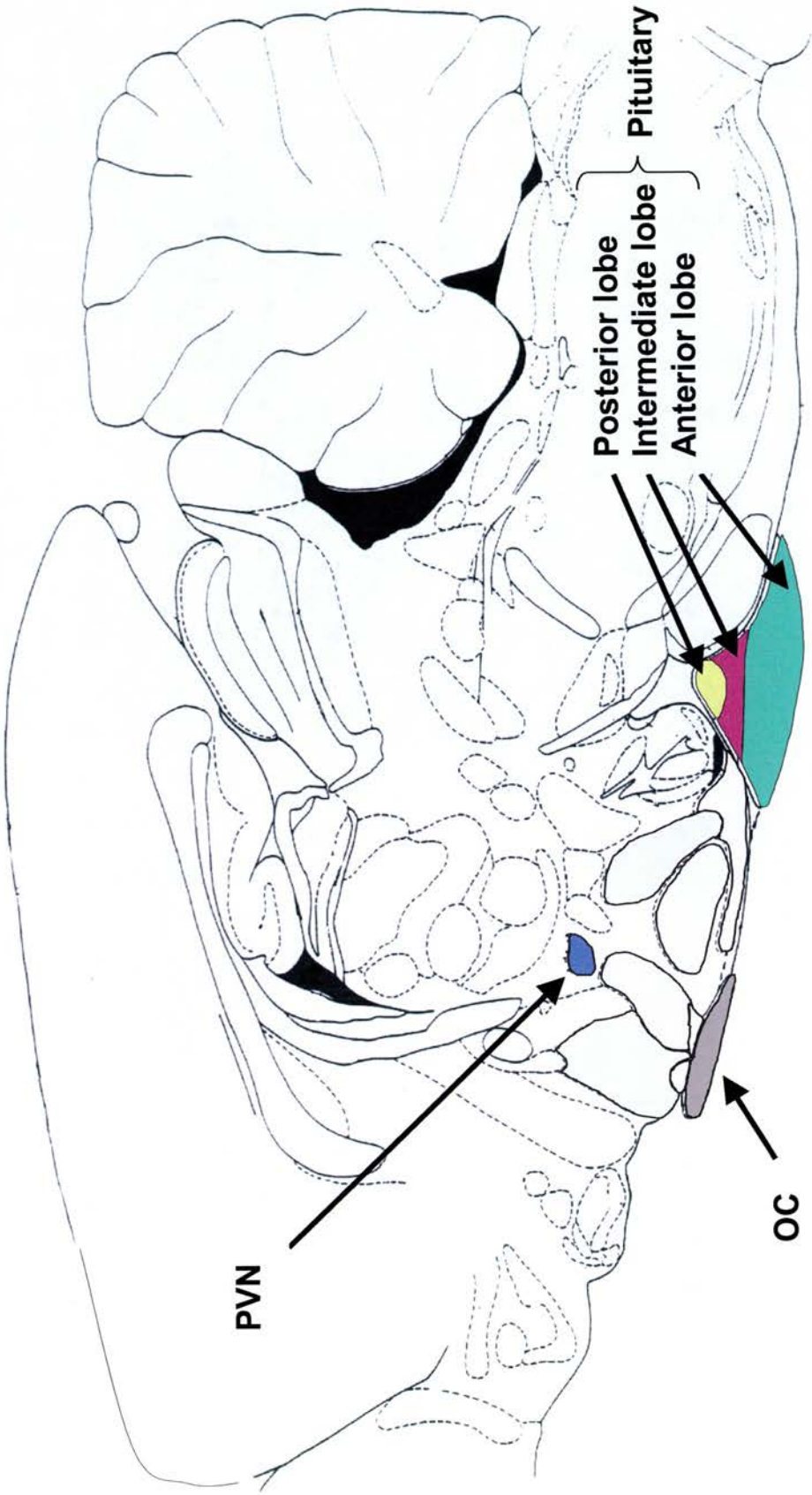


Figure 1.3. Sagittal section through a rat brain.
PVN, paraventricular nucleus; OC, optic chiasm.

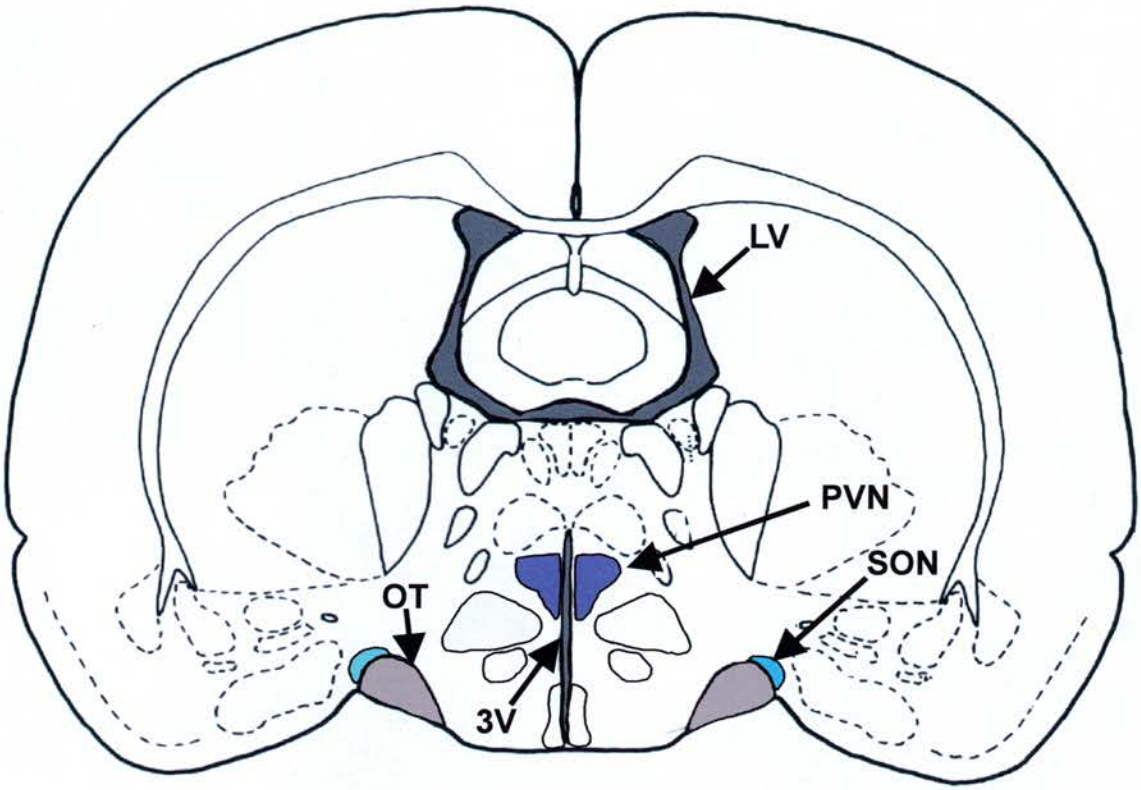


Figure 1.4. Coronal section through a rat brain, showing location of the paraventricular nucleus (PVN).

Section representative of one taken approximately -2.0 mm from Bregma. Abbreviations: PVN, paraventricular nucleus; 3V, third ventricle; OT, optic tract; LV, lateral ventricle; SON, supraoptic nucleus.

1.5.1. The Pituitary Gland

The pituitary gland is also called the hypophysis and is attached to the median eminence of the hypothalamus at the base of the brain. Secretion of the hormones of the pituitary gland is regulated by the hypothalamus. The pituitary gland is divided into three parts: the anterior lobe (*pars distalis*), the intermediate lobe (*pars intermedia*) and the posterior lobe (*pars nervosa*). Together the anterior and intermediate lobes form a true endocrine gland, called the adenohypophysis.

The posterior lobe, also called the neurohypophysis is a neural extension of the hypothalamus. The pituitary is attached to the hypothalamus by the hypophyseal stalk.

1.5.2. The Adenohypophysis

The adenohypophysis is attached to the hypothalamus by the portion of the hypophyseal stalk called the *pars tuberalis* which contains the hypophyseal portal system of blood vessels. The nerve endings of the neurosecretory cells of the hypothalamus terminate at the median eminence, where their hormones are released into the hypophyseal portal system and are then carried to the adenohypophysis. The anterior lobe synthesises and releases six hormones namely adrenocorticotropic hormone (ACTH), growth hormone (GH), follicle stimulating hormone (FSH), luteinising hormone (LH), prolactin (PRL) and thyroid stimulating hormone (TSH), while the intermediate lobe synthesises one hormone: alpha-melanocyte stimulating hormone (α-MSH).

1.5.3. The Neurohypophysis

The neurohypophysis consists of the posterior lobe of the pituitary and the portion of the hypophyseal stalk termed the infundibulum. The infundibulum contains axons of the hypothalamic neurosecretory cells whose cell bodies are located in the magnocellular regions of the PVN and SON of the hypothalamus and synthesise oxytocin and vasopressin.

1.6.1. Pathways Involved in Mediating Stress-Induced HPA Axis Activation

The PVN receives a rich and diverse afferent supply, which are potential routes by which stressful stimuli may influence the HPA axis. Specific central brain circuits drive excitation of the HPA axis in response to stress.

1.6.2. Systemic and Neurogenic Stressors

The stress-processing circuit activated by a particular stressor is dependent upon the nature of the stressor. Generally stress models are divided into two basic categories, referred to as *systemic* (also termed physiological or physical) and *neurogenic* (also referred to as emotional, psychological or processive) paradigms (Fortier C., 1951) (for review see (Sawchenko *et al*, 2000)). Systemic stressors are generally conceived as physiological threats, which are not necessarily consciously appreciated. Systemic stress models commonly employed include cardiovascular, osmotic and immune challenge, as well as ether stress and hypoxia. These stressors have common properties: they are relayed directly to the PVN by visceral afferent pathways and all represent a direct threat to survival. Therefore in these situations it is advantageous to bypass cognitive processing and rapidly relay information to the PVN. In contrast, neurogenic stressors involve sensory processing and a distinct cognitive component. Restraint, immobilisation, novel environment and footshock are often employed as neurogenic stress models. These stressors again have common features: they require processing of signals from somatosensory pathways prior to initiation of a stress response and further, none of the neurogenic stressors represent an immediate threat to homeostasis.

Since lesions to limbic structures (including the prefrontal cortex, hippocampus and amygdala) disrupt HPA responses to neurogenic stressors but have no effect on HPA responses to systemic stressors, Herman & Cullinan (Herman & Cullinan, 1997) have postulated the existence of two generalised stress pathways: "limbic-sensitive" and "limbic insensitive". The former requires processing and integration by the limbic system. The limbic system is then capable of augmenting or attenuated the HPA response depending on prior experience. Limbic insensitive stressors are of immediate survival value and therefore do not require interpretation by the limbic system and instead activate the PVN by a relatively direct pathway.

Transcription of immediate early genes (IEGs) is switched on when a cell is activated and therefore provides a good indicator of neuronal activation. *C-fos*, in particular, has been employed to identify neuronal pathways involved in response to various stressors (Chan *et al*, 1993b) (Hoffman *et al*, 1993). Both neurogenic and systemic stressors activate cells in the pPVN (reflected by *c-fos* induction) (Pezzone *et al*, 1992; Imaki *et al*, 1993; Ericsson *et al*, 1994) with indistinguishable patterns of activation. The patterns of cellular activation in response to these different types of stressor only become distinct when brain areas beyond the PVN are examined.

1.6.3. Brainstem and Medullary Pathways

Brainstem catecholaminergic pathways are known to project directly to CRH producing neurones in the PVN. The nucleus of the solitary tract (NTS) in the brainstem is the principal recipient of sensory information carried by the vagus and glossopharyngeal nerves. Catecholamine synthesising neurones located here project to the PVN both directly and indirectly via the ventrolateral medulla (Cunningham & Sawchenko, 1988; Cunningham *et al*, 1990). Acute stress causes rapid induction of IEG expression in brainstem catecholamine neurones, supporting their involvement in activation of HPA stress responses (Chan *et al* , 1993b; Cullinan *et al*, 1995; Sawchenko *et al*, 1996). Several studies have demonstrated catecholaminergic drive appears to promote HPA activity in response to haemorrhage, hypertension, respiratory distress (Plotsky *et al*, 1989) and immune challenge (Ericsson *et al* , 1994). Further supporting the role of brainstem catecholamine neurones with an excitatory influence on the HPA axis is evidence that deafferentation of ascending brainstem pathways to the PVN inhibits induction of *c-fos* mRNA and Fos protein in pPVN neurones following immune challenge (Li *et al*, 1996). However brainstem deafferentations are ineffective in blocking PVN *c-fos* induction by footshock. These inconsistencies suggest alternative circuitry for different stressors.

Evidence also suggests that ascending aminergic inputs may influence HPA activity following stress. Noradrenergic and serotoninergic inputs from the locus coeruleus (LC) and raphe nucleus respectively, have been implicated in HPA regulation

following haemorrhage (Gand D.S., 1977). However both the LC and the raphe provide fairly limited direct innervation to the PVN (Sawchenko *et al*, 1983; Cunningham & Sawchenko, 1988), thus their effects may be mediated via indirect pathways. The brainstem NTS provides a major noradrenergic input to the PVN (Herman & Cullinan, 1997; Sawchenko *et al* , 2000) and this drive is important in promoting HPA axis activity in response to various stressors including immune challenge and haemorrhage (Plotsky *et al* , 1989; Melik Parsadaniantz *et al*, 1995; Li *et al* , 1996; Day & Akil, 1996).

1.6.4. Forebrain Pathways

Several forebrain structures have been reported to influence the activity of the HPA axis, including the prefrontal cortex, hippocampus, amygdala and septum (Sawchenko *et al* , 1996; Herman *et al*, 1996; Herman & Cullinan, 1997). These brain structures are of particular interest because they are all thought to play some role in emotionality, and may therefore be involved in evaluating or perceiving the stressful nature of a given situation. The amygdala is a limbic brain structure that has been implicated in activation of the HPA axis in response to stress. Stimulation of the amygdaloid complex elicits corticosterone secretion (Dunn & Whitener, 1986) and furthermore large increases in *c-fos* expression have been reported in this brain area in response to restraint and swim stress (Cullinan *et al* , 1995). Consistent with these studies implicating a role for the amygdala in HPA activation, are lesion studies where destruction of the medial or central amygdaloid nuclei, markedly inhibit HPA responses to noise or photic stress (Feldman *et al*, 1994) and similarly lesions to the central amygdala (CeA) significantly reduce the ACTH and corticosterone secretory responses to restraint and fear conditioning (Van De Kar *et al*, 1991). Nevertheless neither medial nor central amygdaloid lesions affect the HPA response to ether exposure (Feldman *et al* , 1994) providing further evidence for stressor-specific pathways.

The bed nucleus of the stria terminalis (BNST) is a limbic structure which links the amygdala and the hippocampus with the hypothalamic and brainstem regions involved in controlling stress responses (Weller & Smith, 1982; Moga *et al*, 1989; Cullinan *et al*, 1993). Stimulation of the BNST evokes corticosterone secretion (Dunn, 1987) whereas, ablation of this region results in attenuated CRH mRNA expression in the PVN and corticosterone secretion, normally induced by conditioned fear (Gray *et al*, 1993; Herman *et al*, 1994).

1.6.5. Hypothalamic Pathways

Most hypothalamic areas have been reported to contribute afferents to parvocellular PVN neurones (Cullinan *et al*, 1996), with the exception of a few nuclei, namely the medial and lateral mammillary nuclei and the supraoptic nuclei. The principal hypothalamic areas providing numerous afferents to the parvocellular PVN include several preoptic areas, the anterior and posterior hypothalamic area, the dorsomedial and ventromedial nuclei and the supramammillary nucleus. Little is known about the precise functions of these nuclei in stress, though they may integrate stimuli perceived by an organism and convey this integrated information to the PVN (Swanson & Sawchenko, 1983).

1.7.1. Stressors

Stressors can be defined either as those that arise suddenly over a relatively short duration: acute stressors, or those which develop over a longer duration and are constantly recurring: chronic stressors.

1.7.2. Acute Stress

A huge range of acute stressors have been used to evoke HPA axis activity including cold, ether, footshock, i.p. hypertonic saline, restraint, forced swimming and surgical stress. All of these challenges lead to an increase in ACTH secretion from the anterior pituitary and increased corticosterone secretion from the adrenal gland. At the level of the hypothalamus acute stress triggers a rapid, but transient increase in immunoreactive CRH in the median eminence, lasting approximately 2-4 minutes (Murakami *et al*, 1989). The time course involved is too rapid to reflect increased CRH synthesis, and instead probably reflects increased transport and/or mobilisation of CRH. Median eminence CRH content begins to decline around 15-30 minutes following the onset of acute stress (Moldow *et al*, 1987), presumably reflecting an increase in release of CRH into the hypophysial portal blood. A second peak in CRH release occurs 60-80 minutes after the onset of the stress (Moldow *et al* , 1987) and it is likely that this represents increased CRH synthesis and transport.

AVP is co-secreted with CRH into the hypophysial portal blood where it stimulates pituitary corticotrope activity (Antoni, 1993). CRH and AVP mRNA expression in the pPVN begin to increase two hours after exposure to an acute stress and peak expression is generally observed at 4 hours after the stressor (Ma & Lightman, 1998). Prior to changes in mRNA expression following stress, CRH and AVP heteronuclear RNA (hnRNA) transcripts in the PVN increase, reflecting rapid gene transcription. Proopiomelanocortin (POMC) is the ACTH precursor and expression of POMC mRNA in the anterior pituitary has been shown to increase in response to acute stressors such as hypertonic saline, forced swimming and restraint (Harbuz & Lightman, 1989).

The importance of the release of CRH and AVP in stimulating ACTH secretion during stress has been demonstrated by immunoneutralisation studies. For example, anti-CRH antiserum substantially reduces the ACTH response to restraint stress, while anti-AVP antiserum is not as effective. However, a combination of both antisera results in a greater reduction in ACTH secretion than either antisera alone (Linton *et al*, 1985).

In summary, acute stress generally results in a rapid release of CRH and AVP from stores in the median eminence and rapid stimulation of gene transcription for these peptides in the pPVN, reflecting increased synthesis. CRH and AVP evoke release of ACTH from the anterior pituitary, which results in a subsequent rise in corticosterone secretion from the adrenal gland.

1.7.3. Chronic Stress

In chronically stressed animals the response of the HPA axis to a homotypic stressor is reduced with each exposure until eventually there is no longer a response (Gomez *et al*, 1996; Akana & Dallman, 1997). The desensitisation of HPA responsiveness to a repeated stress may in part be associated with the desensitisation of the pituitary ACTH response and down-regulation of anterior pituitary CRH receptors, though changes in sensitivity to glucocorticoid feedback may also be involved. Previous studies have shown that although PVN neurones fail to respond (by inducing CRH gene transcription) to a single episode of restraint stress in repeatedly restrained rats, neurones are recruited to produce AVP and there are increased AVP stores in the median eminence (Ma *et al*, 1997a). Habituation (the gradual disappearance of responsiveness to a stressor the animal has become accustomed to) seen in response to repeated stress appears to be stressor specific. Although in chronically stressed rats there is no longer a CRH response to the homotypic stressor the HPA axis remains capable of producing a response to novel acute stressors (Kant *et al*, 1985).

1.8. Corticotropin-Releasing Hormone

1.8.1. Historical Overview

The functional link between the hypothalamus and the adenohypophysis (anterior pituitary) was first proposed by Harris in 1948 (Harris, 1948). The findings of two main studies led to this hypothesis. The first demonstrated activation of the adrenal cortex following electrical stimulation of the hypothalamus and the second showed that lesions to the median eminence disrupted the activity of the adenohypophysis. These observations led him to postulate that factors of hypothalamic origin were released into the hypophysial portal blood and were responsible for controlling the release of anterior pituitary hormones. Direct evidence for the existence and necessity of a hypothalamic "releasing factor" for activation of pituitary corticotrope cells and control of ACTH release was subsequently provided by Guillemin and Rosenburg (Guillemin & Rosenberg, 1955) from experiments performed using *in vitro* pituitary cultures. Since then, the role of the hypothalamus in the regulation of ACTH secretion has been supported by *in vivo* experiments showing that electrical stimulation of the hypothalamus elevates ACTH and corticosterone levels (Dunn & Critchlow, 1973a) and that lesions to the medial basal hypothalamus reduces pituitary-adrenal function (Dunn & Critchlow, 1973b). Over the next 25 years various groups attempted to isolate and characterise the hypothalamic ACTH releasing factor.

Early suggestions that AVP may be the physiological corticotropin-releasing factor were refuted by studies using Brattleboro rats, which do not synthesis AVP, but have essentially normal patterns of ACTH secretion (Arimura *et al*, 1967). Furthermore, other studies demonstrated that hypothalamic fragments have far greater effects on ACTH secretion than vasopressin (Yasuda *et al*, 1976). Besides AVP, several other substances have been reported to stimulate ACTH secretion, including oxytocin, noradrenaline, adrenaline, α-melanocyte stimulating hormone (α-MSH) and β-lipotropin (β-LPH) (Vale & Rivier, 1976). However, for various reasons (Vale *et al*,

1977) none of these known peptides met the criteria expected of the principal hypothalamic corticotropic-releasing factor (CRF), and it became clear that in order to understand the regulation of ACTH secretion, characterisation of the putative CRF was required.

In 1981, Vale and colleagues (Vale *et al*, 1981) finally purified and characterised a 41 amino acid corticotropin-releasing factor (CRH-41) from ovine hypothalamic fragments, and later sequenced rat CRH (Spiess *et al*, 1981). The most definitive evidence for the role of endogenous CRH in controlling ACTH secretion was provided by passive immunisation experiments. Antiserum against CRH was shown to dramatically attenuate plasma ACTH levels in adrenalectomised rats and cause a significant reduction in the ACTH response to stress (Rivier *et al*, 1982). These findings allowed future studies to focus on interactions of factors known to influence ACTH secretion with CRH.

1.8.2. CRH Gene Responses to Stress

Stressful stimuli activate pPVN neurones to stimulate CRH release into the portal bloodstream, which in turn triggers the release of ACTH from the anterior pituitary. CRH neurones respond to acute and chronic stress in different ways.

Acute stress

Exposure to a variety of acute stressors, including restraint, interperitoneal (i.p.) hypertonic saline and forced swimming (Harbuz & Lightman, 1989) has been reported to increase CRH mRNA expression in the parvocellular division of the PVN (pPVN). Generally, a significant increase in CRH mRNA expression is observed 3-4 hours after the onset of stress, for example CRH mRNA is significantly increased 4 hours after the onset of a 60 minute period of restraint (Ma *et al*, 1997b). Measuring levels of CRH heteronuclear RNA (hnRNA; represents primary transcripts and includes precursors of mRNAs from which introns are spliced out) expression better reflects rapid changes in gene transcription. Under basal conditions CRH hnRNA

transcripts are virtually undetectable, however rats killed immediately after a 60 minute period of restraint show significantly elevated levels of CRH hnRNA in the pPVN neurones (Ma *et al* , 1997b). Imaki and colleagues have also demonstrated rapid increases in CRH hnRNA expression in response to restraint increasing within 5 min and a peak in expression at 30 minutes (Imaki *et al*, 1995; Imaki *et al*, 1996b). The cascade of events that mediate the effects of extracellular signals on gene transcription in the PVN is not fully known.

Restraint stress results in rapid induction of immediate early genes (IEGs), including *c-fos*, *jun B* and nerve growth factor induced gene –B (NGFI-B) in the parvocellular division of the PVN (Imaki *et al* , 1996b), all of which are undetectable before stress. Although *c-jun* and *jun-D* mRNA are constitutively expressed in the PVN, small but significant increases in *c-jun* and *jun-D* transcripts are observed 30 min after the onset of restraint stress (Imaki *et al* , 1996b). The time course for induction of these IEG's in response to stress indicates that their products are unlikely to regulate CRH gene transcription, since CRH hnRNA is increased before their transcription. Nevertheless, it is likely that IEGs may participate in the activation of CRH gene transcription. Indeed, the presence of an NGFI-B response element (NBRE) in the promoter region of the rat CRH gene has been reported (Wilson *et al*, 1991).

Chronic Stress

Lightman's group have demonstrated that levels of CRH mRNA in the PVN fail to respond to restraint stress after repeated exposure to daily restraint for 12 days (Lightman & Harbuz, 1993) and actually decline during chronic inflammatory stress (Harbuz *et al*, 1992a). Chronic osmotic stimulation has also been shown to cause a decrease in CRH mRNA in the PVN (Aguilera *et al*, 1993). In chronically restrained rats, exogenous CRH has no effect on plasma ACTH (Hashimoto *et al*, 1988) and pituitary CRH receptors decline (Hauger *et al*, 1990). In repeatedly restrained rats, inhibition of CRH transcription is selective for the homotypic stressor (the same type of stressor) and CRH responsiveness to a heterotypic stress (a different type of stressor, such as i.p. hypertonic saline in this case) is preserved (Ma *et al*, 1999).

Furthermore, exposure of a chronically stressed rat to a novel acute stress elicits a normal (or sometimes enhanced) ACTH response despite the reduction in corticotrope responsiveness due to a reduction in pituitary CRH receptors. Thus repeated stress induces a desensitisation of CRH transcription responses, however rats that have adapted to a chronic homotypical stress retain their ability to respond to a novel heterotypic stressor.

1.8.3. Regulation of CRH gene expression in response to stress

Rapid induction of CRH hnRNA expression (within 5 min) has been reported following exposure to ether stress (Kovacs & Sawchenko, 1996b). This rapid response is incompatible with a primary involvement of any mechanism dependent upon *de novo* protein synthesis. A response element for the immediate early gene, NGFI-B has been identified in the 5' promoter region of the CRH gene (Kovacs & Sawchenko, 1996b), however the rapid induction of CRH hnRNA within 5 min (at least for ether stress) precedes the activation of the NGFI-B gene (which occurs at 30 min) (Kovacs & Sawchenko, 1996a; Kovacs & Sawchenko, 1996b) suggesting that NGFI-B is not involved, at least in response to ether stress, in regulating CRH gene transcription. CRH gene transcription could be mediated by modification of a resident moiety to a transcriptionally active form, such as CREB phosphorylation. CREB is a ubiquitously expressed nuclear protein that mediates transcription by binding to cAMP response elements (CRE) of responsive genes. CREB is phosphorylated (to pCREB) by protein kinase A (Armstrong & Montminy, 1993) or Ca2+/calmodulin-dependent kinases (Hagiwara *et al*, 1993). A CRE has been localised on the CRH promoter (Seasholtz AF, 1988). pCREB induction occurs in the pPVN with a time-course parallel to that of CRH hnRNA expression (following exposure to ether) supporting a role for pCREB in regulating CRH gene transcription.

1.8.4. Central Distribution of Corticotropin-Releasing Hormone

CRH-41 as discovered by Vale and colleagues is the major factor responsible for triggering ACTH release. CRH has been detected by radioimmunoassay in the external zone of the median eminence (Bloom *et al*, 1982) and in the portal blood of rats (Gibbs & Vale, 1982), two areas involved in the transport of hypothalamic factors to the pituitary gland. To determine the source of this CRH, immunohistochemical studies have focussed on the central distribution of CRH. Using antiserum directed against ovine CRH (oCRH), much research has shown CRH is abundantly expressed and widely distributed throughout the rat brain (Olschowka *et al*, 1982; Merchenthaler *et al*, 1982; Bloom *et al* , 1982; Cummings *et al*, 1983; Swanson *et al*, 1983). Further studies using rat CRH antiserum demonstrate similar findings (Rivier *et al*, 1983).

The principal source of CRH involved in control of ACTH secretion can be found in a prominent cluster of approximately 2000 CRH immunoreactive (CRHir) cells in the hypothalamic PVN on each side of the brain (Sawchenko & Swanson, 1985) (see figure 1.5.) Although CRH-stained neurones can be found in each of the subdivisions of the PVN (Swanson & Kuypers, 1980); (for details of PVN subdivisions see section 1.4.) they are most abundant in regions of the parvocellular division (dorso-medial), and these neurones have been shown by retrograde labelling studies to project to the median eminence (Wiegand & Price, 1980a). The localisation of the PVN as the site of CRH cell bodies regulating the anterior pituitary, is consistent with studies of Dunn and Critchlow (Dunn & Critchlow, 1973a) and Palkovitz (Palkovits, 1977) that have emphasised the importance of this hypothalamic area for the regulation of ACTH secretion.

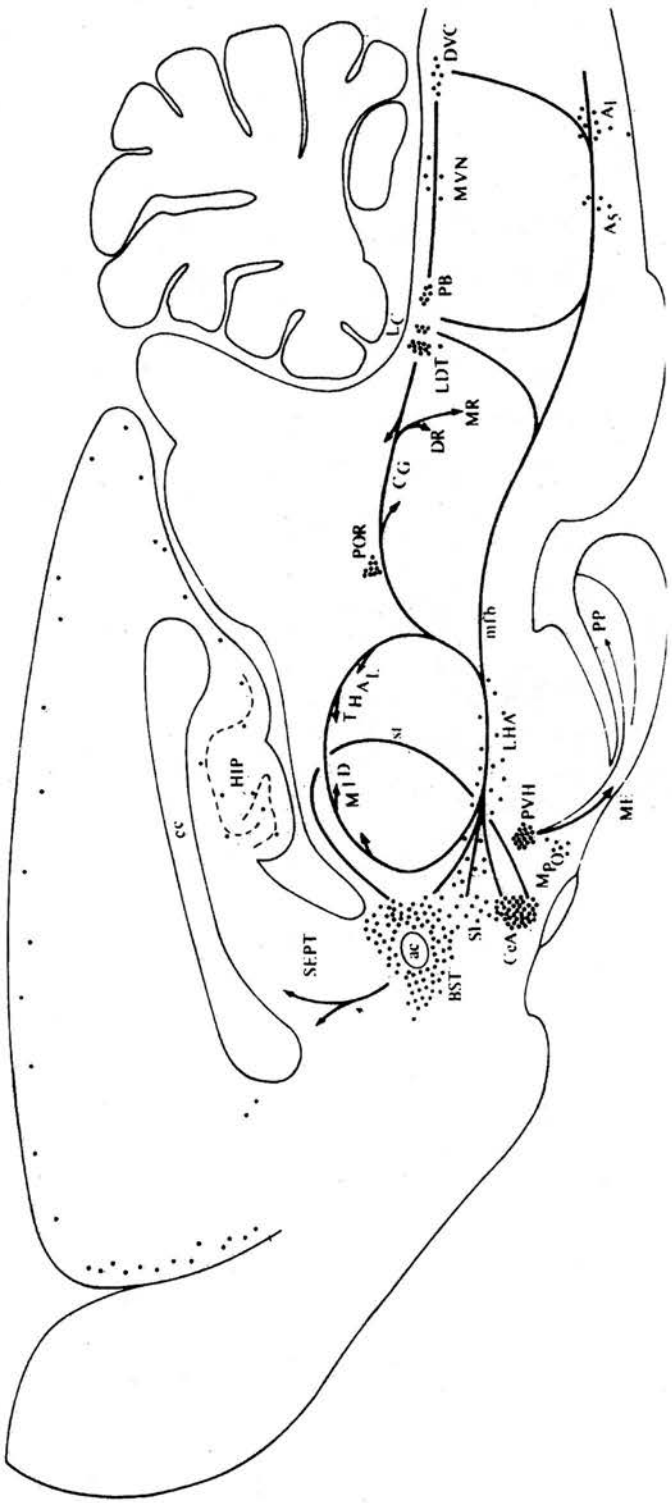

DVC
MVN
PB
LDT
MR
DR
CG
POR
mfb
PP
HIP
MID
LHA
PVH
SEPT
BST
ac
cc

Figure 1.5. Distribution of immunoreactive CRH in the rat brain

CRH Cell bodies (dots) and fibres (lines) are indicated. A prominent group of CRH positive stained neurones are found in the paraventricular nucleus (PVN). Furthermore, a number of cell groups in the basal forebrain and brainstem also contain populations of CRH immunoreactive cells, including the bed nucleus of the stria terminalis (BST), central nucleus of the amygdala (CeA), parabrachial nucleus (PB) and laterodorsal tegmental nucleus LDT). Other abbreviations: ac, anterior commissure; A1 and A5, catecholamine cell groups; cc, corpus callosum; CG, central gray; DR, dorsal raphe; DVC, dorsal vagal complex; HIP, hippocampus; LC, locus coeruleus; LHA, lateral hypothalamic area; mfb, medial forebrain bundle; ME, median eminence; MID THAL, midline thalamic nuclei; MPO, medial preoptic area; MR, median raphe nucleus; MVN, medial vestibular nucleus; POR, perioculomotor raphe region; PP, posterior pituitary; SEPT, septum; SI, substantia innominata; st, stria terminalis (from Swanson, L.W. et al, 1982).

In addition to the cell group that projects to the median eminence, a smaller group of CRH-positive neurones are present in a subset of oxytocin neurosecretory neurones (that project to the posterior pituitary) in the magnocellular division of the PVN and in the supraoptic nucleus (SON) (Burlet *et al*, 1983; Sawchenko *et al*, 1984b). However there is no evidence for co-localisation of CRH with AVP in magnocellular PVN neurones (Roth *et al*, 1983). There are also neurones in the dorsal, medial-ventral and lateral parvocellular subdivisions that express CRH and project to the brainstem and spinal cord. The function of these neurones appears to be in regulating the sympatho-adrenal system (Swanson & Kuypers, 1980). Besides the PVN and SON, other hypothalamic nuclei, including the suprachiasmatic, preoptic and arcuate nuclei (Daikoku *et al*, 1984) also stain positively for CRH peptide.

A number of cell groups in the forebrain and brainstem that are known to be involved in regulation of the autonomic nervous system contain populations of CRHir neurones. Among these are the bed nucleus of the stria terminalis (BNST), central nucleus of the amygdala (CeA), parabrachial nucleus (PB), latero-dorsal tegmental nucleus (LDT) and various brainstem catecholamine cell groups (Swanson *et al* , 1983). CRH stained projections are also present in the two major fibre tracts that inter-connect these areas: the medial forebrain and the periventricular system. This system is a complex one, with the two fibre tracts containing CRH positive ascending and descending projections. While the functional significance remains unclear it is thought that CRH positive cells in these areas may be involved with the central control of autonomic function, and that perhaps these cells mediate the autonomic responses that are complementary to the neuroendocrine actions of CRH.

Brain CRH systems play a role in mediating behavioural responses to stress. The behavioural effects of centrally administered CRH include increased locomotor activity, rearing and grooming (when tested in a familiar environment) (Sutton *et al*, 1982; Dunn & Berridge, 1990b), decreased food intake (Arase *et al*, 1988), decreased sexual behaviour (Sirinathsinghji *et al*, 1983; Sirinathsinghji, 1986) and increased defensive burying (Diamant *et al*, 1992) and decreased exploration in an

open-field (Sutton *et al* , 1982) or elevated plus maze (Baldwin *et al*, 1991). The locus coeruleus, PVN, BNST and CeA are the brain sites predominantly implicated in the behavioural actions of CRH. CRH injected directly into the BNST enhances the acoustic startle response and neurotoxin lesions of the BNST or microinfusion of a CRH receptor antagonist blocks the effects of centrally administered CRH on the startle response (Lee & Davis, 1997). Further, CRH injected i.c.v. potentiates the acoustic startle response (Liang *et al*, 1992) and lesions of the CeA, but not the PVN, block the CRH potentiation of the acoustic startle response (Valentino & Foote, 1987). Micro-injection of a CRH receptor antagonist into the CeA reverses social stess-induced suppression of exploratory behaviour in the elevated plus maze (Heinrichs *et al*, 1992). The PVN may be involved in suppression of food intake. CRH micro-injected into the PVN is effective in decreasing food intake and the CRH receptor antagonist, α-helical CRH, when injected i.c.v. or into the PVN reverses stress-induced suppression of feeding (Krahn *et al*, 1988).

CRH itself could also be involved in the stimulation of transduction pathways within PVN CRH cells to trigger the transcription of the peptide in response to stress. Ono and colleagues first postulated the existence of ultra-short loop positive feedback control of CRH during "alarm states" (Ono *et al*, 1985). Since then, a combined immunocytochemical retrograde tract tracing study in the rat has elegantly demonstrated that the axons of a few CRH neurones in the dorsomedial PVN (which typically display 1-3 dendrites) give rise to terminal boutons around the lateral margin of the PVN (Swanson *et al*, 1987). Thus, CRH-CRH synapses in the PVN, arising from either local connections within the PVN or terminals from other brain areas, could in theory represent CRH auto-regulation in the PVN.

1.9.1. CRH Receptors

The actions of CRH on ACTH secretion are mediated via specific receptors on the plasma membrane of pituitary corticotrope cells. Receptors for CRH have been identified and characterised from rat pituitary glands (Wynn *et al*, 1983). Autoradiographic analysis of the binding of radiolabelled (^{125}I)-CRH in the rodent

pituitary has shown specific receptors to be localised in the anterior and intermediate lobes, with no evidence of binding sites in the posterior pituitary (Aguilera *et al*, 1987). In the rat pituitary, CRH binding is evident as dense clusters in the anterior lobe, corresponding to the distribution of corticotrophs, whereas binding in the intermediate lobe is more uniform, consistent with the homogenously distributed cells known to synthesise proopiomelanocortin (POMC) derived products in this area.

In the brain of both rats and monkeys (Wynn *et al*, 1984; Millan *et al*, 1986), autoradiographic mapping of CRH binding sites has demonstrated localisation in two functionally distinct regions: the neocortex and the limbic system. The highest concentration of CRH binding sites has been localised to regions associated with cognitive function (cerebral cortex), limbic areas associated with emotionality and stress (amygdala, hippocampus and nucleus accumbens) in brainstem regions involved in regulating autonomic function (locus coeruleus and NTS) and in the olfactory bulb, the dorsal horn of the spinal cord and in the cerebellar cortex (De Souza, 1987; Aguilera *et al* , 1987). The presence of CRH receptors in the locus coeruleus (LC) and its multiple connections (to the hypothalamus, hippocampus, cerebral cortex, olfactory bulb, cerebellum and spinal cord, demonstrated by retrograde labelling) suggests that the LC plays an important role in integrating endocrine, autonomic and behavioural aspects of the CNS response to stress.

Electrical stimulation of the amygdala has been shown to cause arousal, attention, fear and rage, reactions associated with sympathetic activation (Kaada, 1972). Similar reactions are observed during stress and after intracerebroventricular (i.c.v.) administration of CRH (Vale *et al*, 1983). Since the amygdala contains CRH and CRH receptors, and furthermore the areas that send afferent projections to and receive efferent projections from the amygdala have all been found to contain CRH receptors, it would seem that CRH may be involved in the generation of the above responses.

In addition, CRH receptors are also found in two hypothalamic areas involved in the control of gonadotropin secretion: the preoptic area and the arcuate nucleus, in both the rat and monkey. Studies demonstrating a reduction in release of luteinising hormone (Rivier & Vale, 1984) and inhibition of sexual behaviour in female rats (Sirinathsinghji *et al*, 1983) after CRH administration into arcuate-ventromedial hypothalamic regions support a role for CRH in regulating sexual function.

1.9.2. CRH Receptor Subtypes

CRH receptors belong to the seven transmembrane G-protein linked receptor superfamily and upon activation stimulate the adenylate cyclase/cAMP signal transduction pathway (Grigoriadis *et al*, 1996). Two major subtypes of CRH receptor (CRH-R) have been identified as the products of distinct genes. The first CRH-R subtype is referred to as the type 1 CRH receptor (CRH-R_1) and the second as the type 2 CRH receptor (CRH-R_2). These receptors share ~70% sequence homology. The CRH-R_1 has been cloned from several species including humans (Chen *et al*, 1993), mouse (Vita *et al*, 1993) and rat (Perrin *et al*, 1993). In each of these species the CRH-R_1 mRNAs encode proteins of 415 amino acids that share 98% homology. While CRH-R_1 generally exists as a single functional form, CRH-R_2 is expressed in three splice variant forms: CRH-$R_{2\alpha}$, CRH-$R_{2\beta}$ and CRH-$R_{2\gamma}$, that differ in their terminal amino termini. Both the CRH-$R_{2\alpha}$ and CRH-$R_{2\beta}$ have been cloned in rats (Vita *et al* , 1993), whereas the CRH-$R_{2\gamma}$ is exclusively found in humans (DeSouza & Kuhar, 1986).

1.9.3. CRH Receptor Distribution

The two different CRH receptors exhibit different distribution in the CNS and pituitary gland. *In situ* hybridisation studies indicate CRH-R_1 mRNA is highly expressed in the pituitary and specific brain regions including several areas of the cerebral cortex, amygdala, cerebellum, hippocampus, brainstem and olfactory bulbs (for reviews see (Grigoriadis *et al* , 1996; Dautzenberg *et al*, 2001)), with very low levels of expression in the hypothalamus of both primates and rodents. In the

periphery, CRH-R_1 mRNA has been found in the adrenal medulla (Palchaudhuri *et al*, 1998) as well as in the testes and ovaries (Vita *et al* , 1993; Nappi & Rivest, 1995). The human placenta has been shown to express CRH-R_1 (Clifton *et al*, 1995; Karteris *et al*, 1998). These binding sites are likely to be involved in regulation of placental CRH (placental CRH is discussed in section 1.19, of this chapter).

The expression patterns of CRH-$R_{2\alpha}$ and CRH-$R_{2\beta}$ in rats are clearly separated between the brain and periphery ((Lovenberg *et al*, 1995). $CRH_{2\alpha}$ mRNA is found in the hypothalamus, lateral septum, BNST, dorsal raphe nucleus (DRN), amygdala and hippocampus, whereas CRH-$R_{2\beta}$ expression in the brain is limited to non-neuronal areas such as the choroid plexus and cerebral arterioles (Dautzenberg *et al* , 2001). CRH-$R_{2\beta}$ is found predominantly in the periphery particularly in the heart, lung, skeletal muscle and the gastrointestinal tract (Dautzenberg *et al* , 2001).

The heterogeneous distribution of CRH-R_1 and CRH-R_2 receptor mRNA suggests distinctive functional roles for each receptor. Within the pituitary, CRH-R_1 expression predominates over CRH-R_2 in both the anterior and intermediate lobes, indicating that type 1 CRH receptors are primarily responsible for CRH-induced changes in ACTH release. Within the lateral septum, very high levels of CRH-R_2 mRNA (Grigoriadis *et al* , 1996) are expressed. This brain region has reciprocal connections with areas throughout the brain associated with learning, memory and autonomic regulation (DeFrance, 1976). Furthermore the septum is involved in the limbic circuitry associated with emotionality such as fear and aggression. The absence of type 1 receptors here suggests a role for CRH-R_2 in regulating limbic circuitry at the level of the septum. In the hypothalamus, CRH-$R_{2\alpha}$ mRNA is highly expressed in the ventromedial nucleus (VMH), supraoptic nucleus (SON) and the medial area of the PVN, SCN and median preoptic area (mPOA) (Grigoriadis *et al* , 1996). Interestingly, within the PVN, CRH-$R_{2\alpha}$ mRNA expression coincides with CRH mRNA expression, which suggests CRH-$R_{2\alpha}$ may act as an autoreceptor in this nucleus. The distribution of CRH receptors in cortical and limbic brain areas correlates nicely with the immunocytochemical localisation of CRH cell bodies and pathways (discussed in section 1.8.4.). Although the mechanisms are not fully

understood the presence of functional CRH receptors in discrete structures of the brain suggests that CRH is involved in regulating CNS function during stress responses.

1.9.4. Regulation of the CRH Receptor

Exposure of the pituitary corticotroph to changes in levels of glucocorticoids, CRH and AVP is thought to play a key role in regulating pituitary CRH receptors (identified as CRH-R_1) during stress. Changes in receptors often parallel responsiveness of the target cell to the hormone ligand. Removal of glucocorticoid feedback by adrenalectomy (ADX) causes increases in hypothalamic CRH and AVP and elevated levels of plasma ACTH (Wynn *et al*, 1985), which results in a marked reduction (~80%) in CRH binding (Aguilera *et al* , 1987) and CRH-R mRNA levels in the rat anterior pituitary (Sakai *et al*, 1996). This is due to a decrease in receptor concentration with no change in binding affinity (Aguilera *et al* , 1987). This receptor desensitisation is an adaptive response to prolonged hormone exposure and has been described in a variety of endocrine systems (Catt *et al*, 1979).

The mechanism responsible for the decrease in pituitary CRH receptor mRNA expression observed after ADX may reflect loss of glucocorticoid actions on the pituitary and/or an increase in hypothalamic secretion of CRH and/or AVP (Plotsky & Sawchenko, 1987; RabadanDiehl *et al*, 1997). Experiments using Brattleboro rats (which lack endogenous AVP), demonstrate that ADX triggers only a small reduction in CRH receptors, compared with an 80% loss in Sprague Dawley and Long Evans rats (Holmes *et al*, 1987), suggesting AVP facilitates the down-regulation of pituitary CRH receptors after ADX.

However, other studies have also demonstrated that corticosterone treatment leads to a reduction in CRH binding, both *in vivo* (Hauger *et al*, 1987) and *in vitro* (Childs & Unabia, 1990) and decreases CRH-R mRNA *in vivo* (Makino *et al*, 1995), although the mechanisms of action are unclear. Administration of corticosterone causes a dose-dependent decrease in the number of CRH receptors and hence CRH binding,

both *in vivo* and *in vitro* (Schwartz *et al*, 1986; Childs *et al*, 1986; Hauger *et al* , 1987) in parallel with a reduction in ACTH secretion (Aguilera *et al* , 1987). This effect of glucocorticoids on CRH receptor down-regulation in the anterior pituitary could be of physiological relevance during acute stress and may participate in the general inhibitory actions of glucocorticoids on ACTH release. However it can only partially account for the inhibitory effects of glucocorticoids on ACTH release since as well as inhibiting CRH stimulated ACTH release glucocorticoids also inhibit the ACTH releasing effects of AVP.

Levels of pituitary CRH receptor mRNA undergo marked alterations during stress, although the changes are dependent upon the stress paradigm used. Using *in situ* hybridisation, receptor binding autoradiography and northern blot analysis, Aguilera's group have demonstrated that following acute hypertonic saline or lipopolysaccharide (LPS) injection there is prolonged decrease in both CRH-R_1 mRNA expression and CRH binding in the pituitary (Rabadan-Diehl *et al*, 1995; Aguilera *et al*, 2001). Whereas acute immobilisation stress (1 hour) results in biphasic changes in pituitary CRH-R_1 mRNA, an initial decrease is followed by recovery and an increase in expression at 4 hours after the onset of the stress, with a concomitant increase in CRH binding (Rabadan-Diehl *et al* , 1995). The mechanism and physiological significance of these responses are not clear, however they may represent differential regulation of pituitary CRH-R in response to emotional and physical stressors.

Several possible mechanisms responsible for down-regulation of CRH receptors have been proposed. One theory is internalisation of receptors following binding to their specific ligands. This hypothesis was supported when radiolabelled CRH was found to be internalised into corticotrophs (Dohanics *et al*, 1991)). Reports of discrepancies in CRH-R numbers and CRH-R mRNA levels suggest post-transcriptional regulation of CRH receptors. A reduction in the binding capacity of CRH-Rs due to uncoupling of the receptor from adenylate cyclase has also been hypothesised (Reisine, 1984).

In contrast to the marked reduction in anterior pituitary CRH receptors, several studies have demonstrated that CRH receptors in the brain are not affected by adrenalectomy (Wynn *et al* , 1984), stress (Aguilera, 1998) or glucocorticoid administration (Hauger *et al* , 1987). However, conflicting evidence was reported more recently. Imaki *et al* demonstrated that both i.c.v. CRH and restraint increase both CRH-R_1 and CRH mRNA expression in the PVN (Imaki *et al*, 1991b; Imaki *et al*, 1996a), suggesting CRH up-regulates CRH-R_1 mRNA in the PVN. This may act to facilitate the positive feedback effects of CRH on its own expression (discussed earlier). Further experiments are required, nevertheless there appear to be differential mechanisms regulating pituitary and brain receptors.

Thus changes in CRH-R expression in the pituitary and/or PVN may affect the responsiveness of the HPA axis.

1.10. Vasopressin

Arginine vasopressin (AVP) is a nonapeptide hormone which was first characterised by du Vigneaud almost 50 years ago (duVigneaud *et al*, 1953). The primary role of AVP is to maintain body fluid homeostasis by regulating blood volume, blood pressure and extracellular fluid levels (Cunningham Jr & Sawchenko, 1991). The source of circulating AVP responsible for these functions is magnocellular neurones in the supraoptic and paraventricular nuclei (SON and PVN, respectively) of the hypothalamus. Axons of these magnocellular neurones project to and terminate in the posterior pituitary, and upon activation release AVP from nerve terminals into the general circulation.

In addition to AVP of magnocellular origin, AVP is also synthesised by parvocellular neurones of the PVN (pPVN). Unlike the magnocellular AVP producing neurones, the parvocellular neurones project to the external zone of the median eminence where they secrete AVP into the pituitary portal circulation. While CRH is potent in stimulating ACTH secretion, AVP alone is a weak ACTH secretagogue (McCann & Brobeck, 1954), however it does play an important role in modulating HPA activity

through its interaction with CRH (Gillies *et al*, 1982). Although magnocellular AVP has been shown under some experimental conditions to gain access to the portal circulation and facilitate pituitary ACTH secretion (Holmes *et al*, 1986a), it is not thought to play a major role in corticotroph regulation.

However, studies have shown that during physiological states involving activation of the HPA axis, such as stress, there is increased expression and secretion of AVP from pPVN neurones (Whitnall, 1989). Under basal conditions, AVP is coexpressed in approximately 50% of parvocellular CRH positive neurones (Whitnall *et al*, 1985) and during exposure to acute or chronic stress this proportion increases substantially (De Goeij *et al*, 1991). CRH and AVP act synergistically to evoke ACTH release both *in vitro* (Gillies *et al* , 1982) and *in vivo* (Rivier & Vale, 1983a). Rivier and Vale reported that pre-treatment of rats with an AVP antagonist attenuates the plasma ACTH response to ether stress and furthermore administration of the same AVP antagonist in conjunction with anti-CRH antiserum completely abolishes ether-stress induced ACTH release (Rivier & Vale, 1983b).

1.10.1. AVP Responses to Acute Stress

In contrast to magnocellular neurones that constitutively express AVP peptide, mRNA and heteronuclear RNA (hnRNA) (Uhl *et al*, 1985), perikarya of the parvocellular neurosecretory neurones do not contain detectable amounts of AVP immunoreactivity, mRNA or hnRNA under basal conditions (Bartanusz *et al*, 1993; Ma *et al* , 1997b). AVP gene transcription is up-regulated by various acute and repeated stressors (Bartanusz *et al* , 1993). For example, a 60 minute period of restraint rapidly induces AVP hnRNA expression in pPVN neurones and is significantly elevated from control levels at 1 and 2 hours after the onset of the stress, whereas AVP mRNA expression is not significantly increased until 4 hours after the onset of the stress (Ma *et al* , 1997b). Similar time courses in AVP gene activation in these cells have been reported after exposure to other stressors, such as ether (Kovacs & Sawchenko, 1996a; Kovacs & Sawchenko, 1996b) and open-field challenge (Priou *et al*, 1993). As well as up-regulated AVP gene expression,

activation of the pPVN neurones stimulates AVP release into the pituitary portal blood system. Upon binding to specific receptors on the pituitary corticotrope cells (see section 1.10.), AVP activates the inositol phospholipid signal transduction pathway and protein kinase C, which results in the mobilisation of Ca^{2+} from intracellular stores and the release of ACTH. AVP does not stimulate POMC transcription like CRH does; however it does accelerate the processing of POMC hnRNA (Antoni, 1993).

1.10.2. Regulation of AVP Gene Expression during Stress

Compared to the time course of stress induced CRH hnRNA levels, which peak within 5 min after an acute challenge (Imaki *et al* , 1995), AVP gene transcription shows a delayed up-regulation in pPVN neurones, suggesting that different mechanisms are involved in regulating the responses of these genes to the same stressors. Regulation of AVP gene expression by glucocorticoids is discussed in section 1.13.3.

The 5'-regulatory region of the AVP gene contains several DNA binding sites or 'response elements' to which transcriptional factors can bind including an NGFI-B response element (NBRE), a CRE and a AP-1 site (Kovacs, 1998). Although the promoter region of the AVP gene possesses a CRE, the onset of AVP gene transcription after stress is delayed by ~ 2h compared to that of CRH and pCREB induction. Other potential candidates for inducing AVP gene transcription during an acute challenge are proteins encoded by immediate early genes (IEG's), such as *c-fos* and nerve growth factor induced gene-B (NGFI-B). Both of these IEG's are induced in parvocellular PVN neurones in response to various stressors (Chan *et al*, 1993a) and as mentioned above response elements (binding sites) for both can be found within the regulatory region of the AVP gene (Chan *et al* , 1993a). Maximal AVP hnRNA expression in pPVN neurones occurs immediately after the peak of expression of transcription factors encoded by IEGs such as Fos and NGFI-B (Kovacs & Sawchenko, 1996b). Further evidence supporting the involvement of IEG encoded transcription factors in stress-induced regulation of AVP gene expression

was provided by Kovacs *et al* (Kovacs *et al*, 1998). They reported that protein synthesis inhibitors (which completely prevent the stress-induced synthesis of IEG encoded transcription factors) significantly attenuated, but did not prevent, AVP hnRNA responses to acute stress. Conversely, protein synthesis blockade did not affect CRH hnRNA levels or phosphorylation of CREB, in response to acute challenge. The *c-fos* promoter itself contains a CRE which binds pCREB (Sassone-Corsi *et al*, 1988). Thus, the mechanisms that regulate AVP gene expression in response to acute stress stimuli may involve both *de novo* synthesis of transcription factors and phosphorylation of CREB.

1.10.3. Chronic Stress and AVP Facilitation

AVP appears to play an important role under chronic stress conditions. Studies have shown that repeated restraint results in increased AVP stores and increased colocalisation of AVP with CRH in pPVN neurones (Bartanusz *et al* , 1993). Plasma corticosterone, and pPVN CRH mRNA and hnRNA levels increase significantly in response to acute restraint stress in rats. These increases become progressively smaller with increasing frequency of exposure to the same stressor, until finally the response to restraint disappears after the rats have been restrained daily for 14 days (Ma *et al* , 1997a; Ma & Lightman, 1998). However, AVP mRNA expression in the pPVN progressively increases as the frequency of exposure to repeated restraint increases (Ma & Lightman, 1998). AVP secretion from nerve terminals in the median eminence has also been shown to increase in response to repeated or chronic stress (De Goeij *et al*, 1992), with enhanced AVP receptor binding in the anterior pituitary (Rabadan-Diehl *et al* , 1995). Furthermore, in repeatedly restrained rats, plasma corticosterone and ACTH levels significantly increase in response to exogenously administered AVP, but do not respond to exogenous CRH (Hashimoto *et al* , 1988).

Schmidt and colleagues have shown that single exposure to a stressor can result in long-lasting changes in AVP stores in the median eminence, and suggested that this may serve as a "stress memory" in adapting responses in the event that the stress is

repeated (Schmidt *et al*, 1995; Schmidt *et al*, 1996). These data suggest that AVP synthesised in the pPVN may play an important role in maintaining HPA axis activity under conditions of chronic stress. As mentioned earlier (in this section), rats which have adapted to repeated stress maintain normal or increased ACTH responses to a heterotypic stressor, and AVP may be an important mediator in maintaining this responsiveness. Since AVP, but not CRH gene responses to repeated stress are preserved, it suggests that the control mechanisms regulating gene transcription and translation are different for AVP and CRH in parvocellular PVN neurones. Thus instead of being considered as a simple potentiator of CRH actions, AVP can also be thought of as an important factor in maintaining HPA responsiveness and in forming "stress memories".

1.10.4. AVP Receptors and AVP Receptor Regulation

AVP secreted by parvocellular neurones of the hypothalamic PVN modulates pituitary ACTH secretion by acting upon AVP receptors on the pituitary corticotrophs. AVP receptors belong to the G-protein linked superfamily. Three major receptor subtypes encoded by different genes have been identified and cloned: (i) V_{1a} receptors, found in smooth muscle and liver are coupled to phospholipase C (PLC) (Morel *et al*, 1992); (ii) V_{1b} (V_3) receptors, expressed in the pituitary and also linked to PLC (Sumimoto *et al*, 1994) and (iii) V_2 receptors, present in the kidney which are coupled to adenylate cyclase (Lolait *et al*, 1992). Regulation of the V_{1b} receptors contributes to the adaptation of the HPA axis to stress.

The cDNA encoding the V_{1b} receptor has been isolated and characterised for human (Sumimoto *et al* , 1994), rat (Lolait *et al*, 1994) and mouse (Ventura *et al*, 1999). The V_{1b} receptor shares only 37-50% identity with the V_{1a} and V_2 type receptors (Lolait *et al* , 1994; Sumimoto *et al* , 1994). The levels of expression of the V_{1b} receptor are highest in the anterior pituitary, however low levels of expression have also been reported in some brain areas and in the periphery (Lolait *et al* , 1994; Saito *et al*, 1995). Northern blot analysis of rat pituitary mRNA reveals two V_{1b} transcripts with molecular sizes of 3.7 and 3.2kb (Lolait *et al* , 1994). The 3.7kb transcript encodes a

biologically active V_{1b} receptor, while the 3.2kb transcript has not been characterised, but appears to be a result of differential splicing or alternative promoter utilisation.

In contrast to pituitary CRH type 1 receptors, a positive correlation exists between pituitary AVP receptor numbers and pituitary ACTH responsiveness. For example, during stress paradigms in which ACTH responses are reduced e.g. chronic osmotic challenge, a down regulation of pituitary AVP receptors is observed, whereas chronic stress protocols which are associated with increases in ACTH responses to novel stressors are associated with up-regulation of AVP receptors (Aguilera, 1994).

Glucocorticoids are elevated during stress (Dallman *et al*, 1987) and thus are likely candidates for regulation of V_{1b} receptor mRNA and AVP binding during activation of the HPA axis. In intact rats, glucocorticoid (dexamethasone) administration decreases AVP binding (Rabadan-Diehl & Aguilera, 1998), despite an upregulation of V_{1b} receptor mRNA levels. This suggests that while glucocorticoids increase V_{1b} receptor mRNA expression by stimulating gene transcription, dexamethasone reduces the number of receptors at the translational and/or post-translational levels. It is difficult to unravel the causes of these changes since the direct effects of glucocorticoid withdrawal on the pituitary are compounded by increased AVP and CRH secretion into the portal blood as a consequence of removing glucocorticoid feedback (Plotsky, 1991). The sustained down-regulation of AVP binding following ADX can be prevented if AVP output is blocked by hypothalamic lesions (Antoni *et al*, 1985); therefore it is feasible that the reduction in AVP binding following ADX is due to an increase in receptor occupancy by AVP. However, administration of corticosterone to rats with hypothalamic deafferentation fully prevents the pituitary AVP receptor loss normally observed (Lutz-Bucher *et al*, 1986), suggesting that glucocorticoids themselves can directly regulate the number of pituitary AVP binding sites. The differential control of AVP receptor number and V_{1b} receptor mRNA may represent a mechanism by which AVP maintains corticotroph responsiveness during stress. Thus changes in V_{1b} receptor expression (for example

as a result of changes in circulating glucocorticoids) will affect the way in which the pituitary (and hence the adrenal gland) responds to stress.

1.11. Oxytocin

Oxytocin is a nonapeptide hormone that differs from AVP by two amino acid residues. It is synthesised by magnocellular neurones in the supraoptic and paraventricular nuclei (SON and PVN, respectively) of the hypothalamus. The axons of these neurones project to the posterior pituitary gland, where oxytocin is stored in vesicles within the nerve endings. Activation of the cell bodies in the hypothalamus results in secretion of oxytocin into the peripheral circulation. The classical physiological functions of oxytocin are associated with female reproductive processes. Oxytocin in the bloodstream participates as a hormone in the control of uterine contractions during parturition and in the milk ejection reflex during lactation (for review see (Leng G, 2000)). Oxytocin, via neuronal projections, also acts on various targets within the brain and modulates maternal, sexual and social behaviours.

In addition to these well-established roles, oxytocin also appears to be a so-called "stress hormone". In 1983 Lang and colleagues showed that in both male and female rats, plasma oxytocin increased 2-3 fold following a 1 minute period of forced swimming and 5-10 fold after a 1 minute period of immobilisation (Lang *et al*, 1983). Since then other studies have reported similar results in response to a range of other acute stressors, including restraint, footshock and ether (for review see (Gibbs, 1986c). A study in 1993 provided evidence for the importance of the hypothalamic PVN for oxytocin release during stress. Denervation of the PVN via anterolateral cuts around the mediobasal hypothalamus (which leaves SON-neurohypophysial connections intact) attenuates oxytocin and ACTH responses to immobilisation stress. Furthermore, surgical lesions that isolate the PVN from autonomic brainstem centres and the SON, abolish the increase in plasma oxytocin and ACTH normally produced by the same stressor (Jezova *et al*, 1993).

The functional relevance of oxytocin release into the peripheral circulation in response to stress is unclear. However, as well as increased circulating oxytocin, high concentrations of oxytocin have also been detected in hypophysial portal blood (Gibbs, 1984) following stress and oxytocin containing nerve terminals have been identified in the external zone of the median eminence (Kawata *et al*, 1983). High concentrations of oxytocin probably reach the anterior pituitary from the median eminence via the portal blood, however some collaterals of the axons of magnocellular neurones may release oxytocin from axon swellings at the median eminence. In the rat, oxytocin has been shown to potentiate the ACTH response to CRH, both *in vitro* (Antoni *et al*, 1884) and *in vivo* (Rivier & Vale, 1985; Gibbs, 1986b). *In vivo*, the stimulatory effect of oxytocin on ACTH secretion has been shown to require the presence of endogenous CRH (Rivier & Vale, 1985). Gibbs reported that immunoneutralisation of oxytocin attenuated the ACTH response to tail hanging stress (Gibbs, 1985). Furthermore he also demonstrated that immunoneutralisation of oxytocin affects ACTH only during stresses associated with increased oxytocin secretion, suggesting that basal levels of oxytocin have no direct or permissive effect on ACTH secretion, and that it is not until oxytocin levels rise in response to stress that the potentiating effects of oxytocin are expressed (Gibbs, 1986a). A more recent study using microdialysis, has demonstrated that oxytocin levels within the PVN increase 7-fold in response to acute stress (Nishioka *et al*, 1998) raising the possibility that local release of oxytocin into the PVN may also play a role in the neuroendocrine stress cascade. Oxytocin does not promote ACTH release through oxytocin receptor receptors in the anterior pituitary, instead it acts via the vasopressin V_{1b} receptor (Schlosser *et al*, 1994).

1.12. ACTH and POMC

Proopiomelanocortin (POMC) is a large polypeptide which contains several bioactive peptide sequences. In the rat, the highest densities of POMC derived peptides are found in the anterior and intermediate lobes of the pituitary gland (DeWied, 1993). POMC is cleaved to form seven peptides: ACTH, α-MSH, β-MSH, γ-MSH, corticotropin-like intermediate lobe peptide (CLIP), γ-lipotropin and β-

endorphin (an endogenous opioid) (Brown, 1998). The conversion of POMC into these active peptides occurs in two stages as shown in figure 1.6. The first cleavage occurs in both the anterior and intermediate lobes, resulting in the production of ACTH and β-lipotropin. In the corticotroph cells of the anterior pituitary the major end product is ACTH. In the melanotroph cells of the intermediate lobe ACTH is further cleaved to produce its final secretory products α-MSH and CLIP, while β-lipotropin is converted to β-endorphin and γ-lipotropin.

In the brain, POMC-containing cell bodies are mainly localised in the arcuate nucleus of the hypothalamus (Emeson & Eipper, 1986). POMC perikarya are also present in the brainstem, in the NTS. In this area α-MSH, CLIP and β-endorphin predominate (Joseph *et al*, 1983). Specialised cells of the anterior piutitary, termed corticotrophs secrete ACTH when signalled by corticotropin-releasing factors (e.g. CRH and AVP). ACTH is a 39 amino acid peptide (Li *et al*, 1943), which binds with melanocortin receptors in the adrenal gland to stimulate secretion of glucocorticoids. These ACTH receptors are part of the G-protein coupled receptor superfamily and once activated increase the production of cAMP via adenylate cyclase (Haynes & Berthet, 1957). Since ACTH is derived from POMC, the processing and availability of POMC in the anterior pituitary corticotrophs determines ACTH output and thus any changes in POMC expression can influence ACTH secretion in response to stress (Young & Akil, 1985).

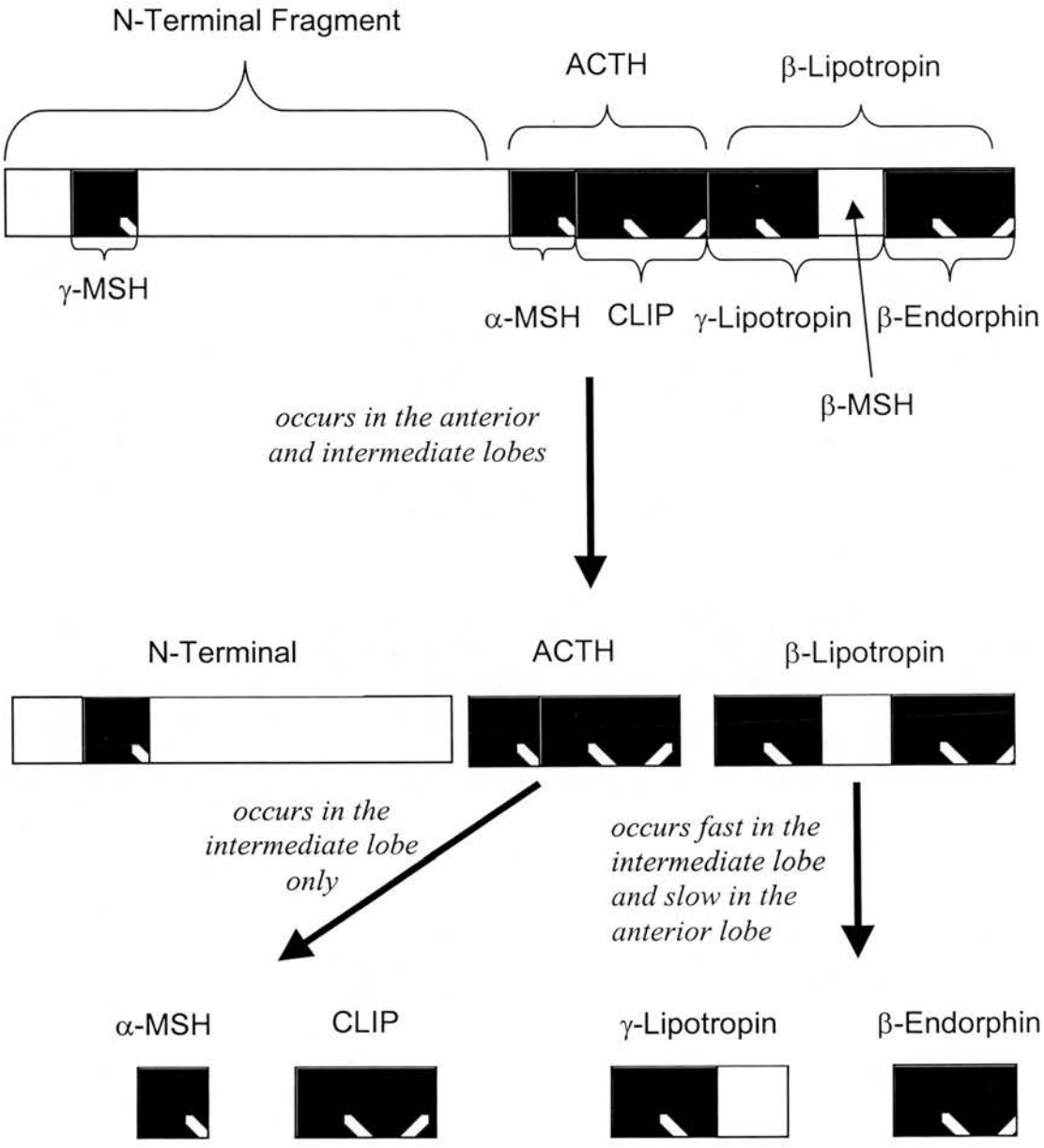


Figure: 1.6. The proopiomelanocortin (POMC) molecule and its conversion to active hormones in the anterior and intermediate lobes of the pituitary gland. MSH, melanocyte stimulating hormone; ACTH, adrenocorticotropic hormone; CLIP, corticotropin-like intermediate factor. Reproduced from Brown, *et al* 1998.

1.12.1. Regulation of ACTH release and POMC gene transcription

The stimulatory actions of CRH and AVP on ACTH have been well established (for review see (Herman *et al* , 1996). Release of CRH and AVP at the median eminence in response to stress stimulates ACTH relese from the anterior pituitary gland. ACTH secretion is also under the inhibitory influence of corticosterone (discussed in section 1.13.3). In addition to acting as a potent ACTH secretagogue CRH has also been shown to be the major factor stimulating POMC gene tanscription in the anterior pituitary (Roberts *et al*,). The exact mechanisms involved in regulating POMC gene expression are not fully understood. *In vitro* studies have shown that CRH stimulation of mouse AtT-20 tumour cells leads to rapid and transient induction of the immediate early gene *c-fos*, accompanied by increased nuclear DNA binding activity of transcription factor, AP-1 (Autelitano, 1994). Furthermore at least part of the mechanism by which CRH stimulates POMC gene transcription in corticotrophs is via a *c-fos* dependent activation of an AP-1 site in the promoter region of the POMC gene (Boutillier *et al*, 1995). *In vivo* studies have also demonstrated that exposure to acute restraint stress induces a rapid increase in *c-fos* and nuclear AP-1 binding activity in the anterior pituitary in parallel with an increase in pituitary POMC gene expression (Autelitano, 1998). These data suggest a role for the immediate early gene, *c-fos* in contributing to increased activity of AP-1 which may be involved in regulating POMC gene transcription in response to stress.

1.13. The Adrenal Cortex and Glucocorticoids

The adrenal glands are located on the proximal poles of the kidneys and consist of two distinct types of tissue: three layers of adrenal cortex surrounding the adrenal medulla. The adrenal cortex is a true endocrine gland which secretes three categories of steroid hormones: mineralocorticoids, glucocorticoids and sex steroids. The adrenal medulla secretes adrenaline and noradrenaline, under the control of the sympathetic branch of the autonomic nervous system. Aldosterone is the primary mineralocorticoid produced by the adrenal cortex. Aldosterone is synthesised in the

zona glomerulosa cells of the adrenal cortex and when released it acts to promote renal reabsorption of sodium. The zona reticularis layer of the adrenal cortex produces small amounts of gonadal steroids, which influence sexual differentiation at puberty.

Glucocorticoids (cortisol in humans, sheep and non-human primates, corticosterone in rodents) are synthesised in the zona fasiculata cells of the cortex of the adrenal gland via a series of enzymatic reactions that utilise cholesterol as the precursor (Campeau *et al* , 1998). ACTH stimulates their synthesis and release during stress. Glucocorticoids play a key role in maintaining homeostasis during stress, they modulate carbohydrate metabolism to free energy supplies and also have anti-inflammatory and immunosuppressive actions. Corticosterone released from the adrenal cortex in response to ACTH completes the negative feedback loop acting at the level of the anterior pituitary, the hypothalamus and other higher brain centres.

1.13.1. Corticosteroid Receptors

The actions of glucocorticoids are mediated via intracellular receptors which belong to the nuclear hormone receptor superfamily. The resulting activated hormone-receptor complex binds to DNA (glucocorticoid response element, GRE) in the cell nucleus and initiates gene transcription and protein synthesis (Munck *et al*, 1990). Some reports have indicated that glucocorticoid receptors occur in the cytoplasm as well as the nucleus (LaFond *et al*, 1988). Two types of receptor for adrenal steroids have been identified (Arriza *et al*, 1987; De Kloet & Reul, 1987; De Kloet *et al*, 1987), Type I (mineralocorticoid, MR) receptors and type II (glucocorticoid, GR) receptors. The two receptors differ in their affinity for ligands (Arriza *et al*, 1988). Type I receptors bind aldosterone, corticosterone, cortisol and deoxycorticosterone equally in vitro. MRs may also bind some synthetic glucocorticoids (e.g. dexamethasone) although with lower affinity (Luttge *et al*, 1989). Type II receptors demonstrate high affinity for synthetic glucocorticoids (e.g. dexamethasone and RU28362) and lower affinity for physiological glucocorticoids (e.g. cortisol and corticosterone) (Reul & De Kloet, 1985). GRs preferentially bind dexamethasone >

cortisol > corticosterone > deoxycorticosterone > aldosterone. MRs have approximately 10-fold higher affinity for physiological glucocorticoids than GRs and in the brain are thought to be involved in controlling basal expression of CRH and AVP at the nadir (Dallman *et al* , 1987) and the peak of diurnal ACTH secretion (Dallman *et al*, 1989). Whereas GRs have a far lower affinity for glucocorticoids and are only activated by high levels of glucocorticoids secreted during stress, and thus are considered to be involved in the control of stress-induced ACTH secretion (Reul & De Kloet, 1985). They provide a mechanism by which negative feedback can terminate stress-activated neural and endocrine (HPA) activation and regulate behavioural responses to stress (McEwen *et al*, 1986). In the periphery, MRs are located primarily in the kidney, whilst GRs occur in liver, muscle and fat cells. However, both MRs and GRs also occur in the brain (Reul & De Kloet, 1985; Reul & De Kloet, 1986).

Apart from regulating gene expression, glucocorticoids can act at the membrane through specific receptors (which have yet to be cloned) to exert multiple rapid effects on various tissues and cells, including nongenomic-mediated feedback inhbition of CRH and ACTH secretion (for review see (Borski, 2000)). Actions at these receptors are rapid since they occur independently of the genome and are transduced by the same biochemical effector pathways resposible for mediating rapid responses to neurotransmitters.

1.13.2. Distribution of Corticosteroid Receptors

Type I receptors have a restricted distribution in the brain, with highest expression confined to the hippocampus, lateral septum, dentate gyrus and brainstem (Reul & De Kloet, 1985; Reul & De Kloet, 1986). The hypothalamus and pituitary gland are relatively devoid of MRs. In contrast, GRs are widely distributed throughout the brain, however they are concentrated in the hippocampus, septum, amygdala, PVN, cortex, brainstem and also in the corticotrope cells of the anterior pituitary. The reason for having two different types of receptors that can bind the same glucocorticoids in the brain is not clear. Evans & Arriza '89 (Evans & Arriza, 1989)

have suggested that a single receptor system cannot respond to the wide range of glucocorticoid levels (from 0.5-100nM), whereas two receptors with different sensitivities could accommodate this range. Thus at low concentrations MRs regulate circadian rhythmicity, while at high concentrations (for example, during stress) GRs regulate stress responses.

Neuronal GR and MR proteins mediate the transcriptional effects of circulating glucocorticoids. These receptors bind to the same DNA response element, yet mediate different cellular functions (Paskitti *et al*, 2000). Corticosteroids enter target cells by passive diffusion where they bind to either GR or MRs. After intranuclear translocation, MR and GR homodimers or corticosteroid receptor heterodimers are formed (Trapp *et al*, 1994), depending upon the relative concentrations of both receptors. The different corticosteroid receptor dimers bind to glucocorticoid response elements (GREs) in the flanking regions of target genes (CRH (Malkoski & Dorin, 1999), AVP (Kim *et al*, 2001) and POMC (Drouin *et al*, 1987) all contain GREs in their promoter regions) where they can regulate gene transcription.

1.13.3. Glucocorticoid Feedback Regulation of the HPA axis

The HPA axis is sensitive to feedback inhibition by corticosterone, under basal and stress conditions. Many studies investigating glucocorticoid feedback have employed adrenalectomy (ADX). Complete removal of glucocorticoid feedback by bilateral ADX produces a rapid increase in ACTH secretion, within 20 minutes (Dallman *et al*, 1972). At the PVN, CRH and AVP mRNA expression increases in parvocellular neurones (Wolfson *et al*, 1985; Jingami *et al*, 1985), as does CRH and AVP peptide expression (Swanson *et al* , 1983; Sawchenko *et al*, 1984a). The AVP-CRH ratio also increases (Holmes *et al*, 1986b), suggesting that the signal to the pituitary for ACTH release is enhanced. At the pituitary, POMC mRNA expression increases 4 hours after ADX (Eberwine & Roberts, 1984) and during the first 24 hours after ADX, pituitary ACTH content and pituitary corticotrope cell numbers double (Rappay & Makara, 1981). Within several days the rate of ACTH synthesis is significantly increased, as are ACTH stores (Birnberg *et al*, 1983). All of these

adaptations induced by ADX can be reversed by glucocorticoid treatment, indicating that they are a consequence of disrupting the negative feedback loop.

The negative feedback effects exerted by glucocorticoids have been shown *in vivo* and *in vitro* to occur over at least three distinct time domains: rapid (seconds to minutes), intermediate (minutes to hours) and slow (hours to days) (for review see (Keller-Wood & Dallman, 1984)).

1.13.3.1. Rapid Glucocorticoid Feedback

Rapid (or fast, rate-sensitive) feedback was first postulated by Dallman & Yates (Dallman & Yates, 1969). Administration of corticosteroids to rats inhibits the corticosterone response to histamine administration when the injection precedes histamine by 15 seconds or 5 minutes, however not if the corticosterone is administered 15 minutes before or 2 minutes after the administration of histamine (Dallman & Yates, 1969). The authors suggested that this rapid feedback occurs when plasma corticosterone levels are rising and suggested that this feedback is rate sensitive. Later, Kaneko and Hiroshige (Kaneko & Hiroshige, 1978) demonstrated that the rate of rapid corticosterone feedback was dependent upon the rate of association of corticosterone with its receptor. Rapid glucocorticoid feedback inhibition has been demonstrated to have rapid effects on both stimulated CRH (Vermes *et al*, 1977) and ACTH secretion (Buckingham & Hodges, 1977; Vale & Rivier, 1977). However, this rapid inhibition is not affected by pretreatment with cycloheximide (a protein synthesis inhibitor) (Widmaier & Dallman, 1983a), indicating that the rapid feedback action of corticosterone does not require protein synthesis. Furthermore, fast corticosterone feedback does not affect ACTH content in pituitaries (Widmaier & Dallman, 1983b) or CRH content in the hypothalamus, indicating that the secretory process, but not the synthetic process is inhibited by corticosteroids (Jones & Hillhouse, 1976; Jones *et al*, 1977).

1.13.3.2. Delayed Glucocorticoid Feedback: Intermediate and Slow

Many studies have demonstrated delayed feedback by corticosteroids on stress-induced activity of the HPA axis (Sayers & Sayers, 1947; Smelik, 1963; Hodges & Sadow, 1967; Dallman & Yates, 1969). Infusions of corticosterone beginning 120 minutes before histamine administration inhibit the endogenous corticosterone response to histamine, however, infusions beginning between 10-45 minutes before the histamine administration have no effect on the response (Dallman & Yates, 1969). The authors hypothesised that this represents a delayed feedback effect of corticosterone levels at the time of stress, which requires between 45-120 minutes to develop. The effectiveness of corticosteroid administration appears to be dependent upon the levels of steroid achieved (Sayers & Sayers, 1947; Dallman & Yates, 1969). Takebe and colleagues demonstrated that the extent and duration of corticosterone inhibition depends on the dose administered (Takebe *et al*, 1971). A range of studies have demonstrated that the maximal inhibition of the adrenocortical responses occur between 2-4 hours after administration of a single dose of corticosterone (Smelik, 1963; Takebe *et al* , 1971; Jones *et al*, 1974). The inhibitory effect is attenuated 6-12 hours after administration of corticosterone, however extremely high doses of corticosterone prolong the duration of inhibition (Jones *et al* , 1974), as does repeated corticosterone administration. Others have reported that as well as the dose, the duration of corticosterone exposure also determines the period of inhibition of the adrenocortical axis (Abe & Critchlow, 1980). Sustained elevation of glucocorticoids for one or more days abolishes the capacity of the HPA axis to respond to various stimuli (Buckingham & Hodges, 1974; Engeland *et al*, 1975). Thus an important factor in delayed feedback appears to be the duration of exposure to enhanced levels of corticosterone. Incubation of AtT-20 cells (a murine pituitary tumour cell line) with dexamethasone (a synthetic glucocorticoid) for 5-25 hours causes a significant reduction in ACTH release, however following 2-3 days of dexamethasone exposure, as well as a reduction in ACTH secretion, ACTH content is also significantly attenuated. Moreover, glucocorticoid administration for 3 days not only decreases pituitary ACTH content (Keller-Wood & Dallman, 1984) but also the number of

pituitary corticotrophs (Keller-Wood & Dallman, 1984). Together these data indicate that prolonged exposure to corticosterone severely inhibits synthesis and secretion of ACTH (Keller-Wood & Dallman, 1984). These findings led to the theory that delayed feedback regulation by glucocorticoids exert distinct effects over time. Subsequently delayed feedback inhibition was designated either "intermediate" for corticosteroid feedback that results from 2-10 hours of prior corticosterone exposure and lasts for relatively short durations; or "slow" for feedback inhibition that results from constant corticosterone exposure for 12 or more hours and persists for relatively longer durations. Furthermore, intermediate corticosterone feedback inhibits ACTH release, but not synthesis, whereas slow feedback inhibits both. Nevertheless, intermediate feedback requires DNA-dependent RNA synthesis, since the inhibitory actions of corticosterone on ACTH secretion can be prevented by pre-incubation of pituitaries with the RNA polymerase inhibitor, actinomycin D (Portanova & Sayers, 1974). In addition, intermediate feedback also influences CRH synthesis and release. CRH bioactivity in the hypothalamus, and basal and stimulated CRH release are all increased in ADX rats, an effect which can be reversed by corticosterone replacement (Vernikos-Danellis, 1965; Sato *et al*, 1975; Hillhouse & Jones, 1976; Buckingham, 1979). Since CRH content is not affected (Jones & Hillhouse, 1976), it would seem that CRH synthesis is inhibited as well as CRH release.

Slow corticosterone feedback (exposure to glucocorticoids for 24 hours or more) inhibits ACTH release (Engeland *et al* , 1975), ACTH synthesis (Keller-Wood & Dallman, 1984) and reduces POMC mRNA expression (Schachter *et al*, 1982). In summary, intermediate feedback appears after relatively short durations of corticosterone exposure, whereas slow feedback becomes evident following prolonged periods of high circulating plasma corticosteroids. Hence rapid and intermediate feedback would be expected under conditions where increases in plasma corticosterone occur in response to stress, whereas slow feedback would be expected when elevated plasma levels of corticosterone persist for days.

1.13.3.3. Sites of Glucocorticoid Feedback

There is evidence for glucocorticoid feedback at the level of the anterior pituitary, hypothalamus and other higher areas in the brain. Injection of corticosterone or dexamethasone directly into the pituitary decreases the corticosterone response to stress (Russell *et al*, 1969). Administration of corticosterone or dexamethasone to animals with median eminence or hypothalamic lesions (Jones *et al* , 1977) also results in attenuated corticosterone responses. Further evidence for feedback at the level of the pituitary has been provided by in vitro studies: corticosterone inhibits stimulated ACTH release from various pituitary preparations, including monolayer cultures (Fleischer & Rawls, 1970), isolated pituitary cells (Portanova & Sayers, 1974), perfused pituitaries (Widmaier & Dallman, 1983b; Widmaier & Dallman, 1984) and cultured AtT-20 cells (Herbert *et al*, 1978). The hypothalamus has been implicated as a site of corticosteroid feedback since local administration or implants of corticosterone in the median eminence or antero-medial hypothalamus effectively inhibits pituitary-adrenal activity (for review see (Keller-Wood & Dallman, 1984)), however this evidence is not definitive as implanted corticosterone may enter the portal blood and exert its actions at the anterior pituitary. Nevertheless, both basal and stress-induced CRH bioactivities within the hypothalamus are inhibited by dexamethasone or corticosterone administration (Takebe *et al* , 1971; Buckingham & Hodges, 1977; Kaneko & Hiroshige, 1978) and CRH release into the portal blood system in response to haemorrhage is inhibited by dexamethasone pretreatment (Plotsky & Vale, 1984) supporting the hypothalamus as a site for glucocorticoid feedback regulation of the HPA axis. Corticosterone feedback has also been reported at extra-hypothalamic sites in the brain, particularly at limbic brain structures. Injection of corticosterone into the septum or hippocampus suppresses the adrenal response to various stimuli (Keller-Wood & Dallman, 1984).

Several researchers have attempted to identify the receptors involved in glucocorticoid feedback. Reul & deKloet reported that type I (high affinity) receptors are 90% occupied during the normal circadian trough, when basal levels of

corticosterone are low; whereas type II (low-affinity) receptors are less than 5% occupied at this time and approximately 50% occupied at the circadian peak or following stress in the morning when corticosterone levels are elevated (Reul & De Kloet, 1985). Since type I receptors are restricted primarily to the lateral septum and hippocampus and because they are essentially fully occupied by corticosterone (Dallman *et al*, 1994), their role may be permissive. In contrast, the type II receptors have a wider distribution, with high expression in the lateral septum, dentate gyrus, NTS, CeA and PVN, and furthermore are only 50% maximally occupied at the circadian peak and after stress during the trough phase. Thus the characteristics of type II receptors indicate they are more suited to feedback regulation of the stress-induced increases in ACTH secretion

1.13.4. Corticosterone Binding Globulin

Corticosterone binding globulin (CBG) is synthesised in the liver of most mammals including rodents and humans (Hammond *et al*, 1987). CBG is a carrier protein found in plasma which (as its name suggests) binds corticosterone. Between 90-95% of circulating corticosterone becomes bound to CBG under normal conditions (Hammond, 1990). Since corticosterone is active only in its free form (Mendel, 1989), there is normally only a small fraction of total corticosterone available for bioactivity. Therefore, any variations in CBG levels can strongly influence the glucocorticoid signal. Glucocorticoids are known to influence plasma CBG levels as does stress. Removal of endogenous glucocorticoids by ADX increases circulating levels of CBG (Weidenfeld & Feldman, 2000), whilst chronic corticosterone administration causes plasma CBG levels to decline (Weidenfeld & Feldman, 2000). Similarly, increases in plasma corticosterone following exposure to acute stress also significantly reduce plasma CBG levels (Fleshner *et al*, 1995; Spencer *et al*, 1996). Thus circulating CBG provides a mechanism by which the availability of biologically active glucocorticoids may be regulated without necessarily altering total levels. 11β-hydroxysteroid dehydrogenase (11β-HSD) may represent another important factor in the control of corticosteroid feedback and is discussed in section 1.21.

1.14. Circadian Rhythm of HPA Activity

Under normal conditions, many mammals including rats and humans display a diurnal pattern of ACTH and corticosterone secretion (Krieger, 1977). The peak and nadir of hormone secretion coincides with the initiation and termination of the animal's active period, respectively. Rats are nocturnal animals, therefore plasma levels of ACTH and corticosterone peak in the evening and are lowest in the morning (Dallman *et al*, 1978). Plasma ACTH and corticosterone levels rise throughout the afternoon, however the peak in ACTH secretion precedes the peak in corticosterone secretion. In rats (entrained to a 12 hour light-dark cycle, lights on at 06:00h) corticosterone levels are very low during the early morning (06:00-09:00h) and they increase slightly between 11:00 and 13:00h. Corticosterone levels begin to rise dramatically in the late afternoon, and peak at around two hours after the onset of the dark phase (~20:00h). Plasma corticosterone then gradually declines reaching near basal levels at 03:00h (Kwak *et al*, 1992). Rats also exhibit low plasma ACTH levels in the morning. ACTH levels increase between 13:00-15:00h and peak just before the dark phase at 17:00h (Kwak *et al* , 1992). ACTH levels decline rapidly ~ 1 hour after the peak in corticosterone secretion and return to near basal levels at ~03:00h.

CRH is the principal regulator of ACTH secretion and its presence is necessary for a normal circadian rhythm of ACTH. Immunoneutralisation of CRH abolishes the evening rise in plasma ACTH and corticosterone levels (Bagdy *et al*, 1991). CRH mRNA content in the hypothalamus also exhibits diurnal variations. Generally, CRH mRNA content is low in the morning, begins to rise in the afternoon and peaks at the onset of the dark phase (Kwak *et al* , 1992). Following this peak in the evening there is a sharp decline in CRH mRNA content, which coincides with the peak of corticosterone secretion, and then a second drop occurs early in the morning, when CRH mRNA expression is minimal (Kwak *et al* , 1992).

The suprachiasmatic nucleus (SCN) of the hypothalamus is thought to play an important role in generating the HPA diurnal rhythm, as bilateral lesions to the SCN

significantly attenuate (Abe *et al*, 1979) or completely abolish (Moore & Eichler, 1972) the peaks in ACTH and corticosterone secretion. Furthermore, the serotoninergic innervation of the SCN neurones seems to be important in facilitating the ACTH rhythm. Lesioning of the median and dorsal raphe nuclei (the site of serotonin producing neurones) causes a marked depression (~40-50% reduction) in the amplitude of ACTH secretion (Szafarczyk *et al*, 1980). It is not clear how the SCN controls the circadian rhythm of adrenocortical function, however anterograde tracing techniques have shown that some SCN neurones innervate the dorsomedial parvocellular PVN cells (Stephan *et al*, 1981). Other lesioning studies have demonstrated that the SCN stimulates CRH secretion at night (Cascio *et al*, 1987), supporting the idea that the SCN-PVN pathway is important in regulating the circadian rhythm of ACTH secretion.

As well as the input from the SCN, CRH neurones in the PVN also receive catecholaminergic innervation from the locus coeruleus and the A1 and A2 cell groups in the brainstem. These brainstem projections have been implicated in modulating the diurnal HPA rhythm (Kaneko *et al*, 1980), since destruction of the ventral noradrenergic ascending bundle obliterates the circadian pattern of ACTH and corticosterone.

The expression of so-called "pacemeaker" or "clock" genes in the SCN, including the genes *per* (period) and *tim* (timeless) are fundamental in generating 24h rhythmicity. The protein products of these genes act through a negative feedback loop to repress their own transcription. This time-keeping mechanism relies on two factors: lag between transcriptional induction of *per* and *tim* and the nuclear translocation of the repressor proteins they encode. The interaction of these genes with transcriptional activators are thought to play a crucial role in the molecular machinery that drives physiological and behavioural rhythmicity (for review see Wager-Smith & Kay, 2000).

1.15. Opioids and the HPA axis

Endogenous opioids and their receptors are widely distributed throughout the CNS. Their distribution is consistent with their involvement in a broad range of functions and behaviours, including regulation of pain, reinforcement and reward, release of neurotransmitters, autonomic and neuroendocrine modulation (Akil *et al*, 1984; Olson *et al*, 1995). Three families of endogenous opioid have been identified: the enkephalins, dynorphins and endorphins, each derived from different precursors: proenkephalin (pENK), prodynorphin (pDYN) and proopiomelanocortin (POMC), respectively (Drolet *et al*, 2001). Related to the opioid family is the recently discovered peptide orphanin FQ (OFQ)/ nociceptin (Reinscheid *et al*, 1995; Meunier *et al*, 1995).There are three main types of opioid receptor, all belonging to the G-protein linked receptor superfamily, referred to as mu (μ), delta (δ) and kappa (κ) (Mansour *et al*, 1994). Enkephalins bind to μ- and δ-receptors, dynorphins to κ-receptors and endorphins to μ- and κ- receptors (Russell & Douglas, 2000). Opioid synthesising neurones are present in stress related regions of the brain and brainstem, including the cerebral and piriform cortex, central and medial amygdala, lateral septum, BNST, preoptic area, hypothalamus (including the PVN), parabrachial nucleus, NTS and VLM (Khachaturian *et al*, 1983; Harlan *et al*, 1987). Opioid receptors are present in the median eminence and there are opioidergic inputs to the PVN (Drolet *et al* , 2001). β-endorphin cells in the arcuate nucleus send projections which traverse the median eminence and terminate in the PVN. These β-endorphin nerve terminals impinge upon CRH neurones in the PVN and therefore may be involved in controlling CRH release. Furthermore, enkephalins and dynorphins are co-synthesised by noradrenergic neurones in the NTS which are known to project to the PVN (for review see (Russell & Douglas, 2000)). Activation of these NTS neurones which may co-express opioid peptides (pENK-A and pDYN mRNA) occurs following exposure to stress (Russell & Douglas, 2000).

Immunocytochemical studies have revealed the coexistence of enkephalin and CRH in pPVN cells (Hokfelt *et al*, 1983) and of enkephalin, CRH and AVP in granules in

the median eminence (Hisano *et al*, 1987), suggesting a role for opioids in HPA axis regulation, perhaps by a pre-terminal action in the median eminence. Expression of pENK-A mRNA is increased in the PVN after exposure to various stressors, such as i.p. hypertonic saline (Lightman & Young, 1987; Harbuz & Lightman, 1989), ether (Watts, 1991), morphine withdrawal (Harbuz *et al*, 1991) or restraint (Ceccatelli & Orazzo, 1993). However, the stress-induced increase in pENK-A mRNA in the PVN is blocked by i.c.v. administration of a GR antagonist (Garcia-Garcia *et al*, 1998) indicating that the increase may be a result of stress-induced glucocorticoid action on these neurones (or their inputs). Thus opioids may influence HPA activity by their actions at the level of the brainstem, PVN or the median eminence.

Various studies have indicated that opioid peptides play both stimulatory and inhibitory roles in regulating HPA activity. Acute administration of morphine causes a rise followed by a fall in hypothalamic CRH content and increases concentrations of ACTH in plasma and in the anterior pituitary *in vivo* (Buckingham, 1982). Furthermore, stress-induced increases in hypothalamic CRH and pituitary and plasma ACTH levels are potentiated in rats pretreated with morphine (Buckingham, 1982). Incubation of isolated hypothalami with either morphine, met-enkephalin or leu-enkephalin all stimulate CRH secretion, effects which can be inhibited by naloxone (Buckingham, 1982); however, none of these opioids has any effect on ACTH secretion from pituitary segments *in vitro* (Buckingham, 1982). These data suggest that morphine and the enkephalins evoke HPA activity by stimulating specific receptors in the hypothalamus. In contrast, hypothalami of morphine tolerant rats fail to secrete CRH when stimulated with either morphine or met-enkephalin (Buckingham & Cooper, 1984) both *in vivo* and *in vitro*. Morphine also inhibits stress-induced ACTH secretion when given acutely to rats anaesthetised with sodium pentobarbitone (Buckingham, 1982). β-endorphin has been shown to stimulate and inhibit CRH secretion(Buckingham, 1986). At low concentrations, β-endorphin causes dose-related increases in CRH release from rat hypothalami *in vitro*, whereas at high concentrations, β-endorphin inhibits CRH release (Buckingham, 1986). The stimulatory response is inhibited by naloxone, whereas the inhibitory response is not readily reversed (Buckingham, 1986), indicating that each response is mediated by

different receptors. The opposing actions of endogenous opioids in regulating HPA function are thought to be mediated by specific opioid receptor subtypes in the hypothalamus.

In rodents the ACTH response to acutely administered morphine can be mimicked by selective μ-receptor agonists and inhibited by naloxone, naltrexone and the selective μ-receptor antagonist, β-funaltrexamine (Pfeiffer *et al*, 1985; Hayes & Stewart, 1985; Pechnick *et al*, 1985a; Pechnick *et al*, 1985b; Buckingham & Cooper, 1986). Blockade of μ-receptors has no effect on basal ACTH or corticosterone levels, however the secretion of these hormones normally observed in response to surgical stress is abolished (Cover & Buckingham, 1989), suggesting that these receptors may facilitate the secretion of CRH that occurs in response to certain stressors. δ-receptors do not appear to be involved in the regulation of the HPA axis, since autoradiographic studies indicate the hypothalamus is almost completely devoid of δ-binding sites (Mansour *et al*, 1987) and the δ-receptor antagonist has no effect on ACTH and corticosterone responses to stress (Cover & Buckingham, 1989). Blockade of κ-receptors evokes a rapid hypersecretion of basal ACTH and corticosterone and causes a pronounced exaggeration and prolongation of the stress response (Cover & Buckingham, 1989). Furthermore, the hypothalamus is rich in κ-binding sites (Mansour *et al* , 1987) and κ-agonists have been shown to depress the secretion of immunoreactive CRH *in vivo* and *in vitro* (Yajima *et al*, 1986; Plotsky *et al*, 1987), indicating a role for κ-receptors in tonic inhibition of the HPA axis.

In the rat, stress stimulates oxytocin secretion. Endogenous opioids play an important role in the control of oxytocin release (Clarke *et al*, 1979) and in the rat, have been shown to exert an inhibitory influence. Oxytocin neurones of the SON and PVN co-express enkephalins and dynorphins and upon activation, opioids are co-released with oxytocin (for review see (Brown *et al*, 2000)). κ- and μ-receptors are expressed on oxytocin cell bodies in the magnocellular region of the PVN and in the SON, however only κ-opioids have been identified on nerve terminals in the posterior pituitary (Russell & Douglas, 2000). Since no evidence has demonstrated the

presence of δ-receptors in the hypothalamo-neurohypophysial system (HNS), it is thought that opioids acting upon δ-receptors are not of primary importance in regulating oxytocin secretion in response to stress. The oxytocin secretory response to various stressors (such as forced swimming and restraint) is increased by naloxone and inhibited by κ- and μ-opioids. In the rat, endogenous opioids to inhibit secretion by acting on oxytocin axon terminals in the posterior pituitary (Douglas *et al*, 1993), however, exogenous opioids can act directly on oxytocin cell bodies to inhibit their activity via κ- or μ-receptors (Douglas *et al*, 1995).

A sexual dimorphism in the oxytocin response to stress exists (Carter *et al*, 1986). Immobilisation stress causes a significant increase in plasma oxytocin concentration in both intact male and female rats and in castrated males, however this response is significantly potentiated by naloxone only in female and castrated male rats (Carter *et al* , 1986). These results indicate that an inhibitory opioid mechanism exerts a more profound influence on oxytocin secretion in female than in male rats and suggests that gonadal hormones may modulate the opioid system regulating neurohypophysial oxytocin secretion. Thus, endogenous opioids play important modulatory roles in regulating both HPA and HNS activity in response to stress.

1.16. Interactions of the Neuroendocrine and Immune Systems

The importance of the immune and neuroendocrine systems for successful recovery from infection or inflammation is well established. The immune system recognises and eliminates foreign antigens, regulates inflammatory processes and plays a key role in tissue repair. The neuroendocrine system ensures that the altered metabolic demands of the host are adequately met. These two systems do not function independently. The immune and neuroendocrine systems interact to maintain homeostasis. Until recently most interactions between the immune and neuroendocrine system were attributed to the immunosuppressive action of glucocorticoids, however it is now well recognised that the immune-neuroendocrine interactions are bidirectional. In addition to the effects of hormones on the immune system, immune signals profoundly affect neuroendocrine functions.

1.16.1. Neuroendocrine Effects on the Immune System

The anti-inflammatory effects of glucocorticoids were discovered in the early 1950's (Hench & Kendall, 1950). The ultimate effect of adrenal glucocorticoids is to suppress immune/inflammatory responses. They suppress lymphocyte traffic and function, the secretion of cytokines and other inflammatory mediators and inhibit the effects of several of these mediators on their target tissues (Blalock, 1989), so as to prevent compromising homeostasis. Under normal conditions glucocorticoids exert a tonic inhibitory influence on the immune system, however they are not present in large enough quantities to prevent the organism mounting an immune response to a challenge. As the hypersecretion of corticosterone is dependent upon the immune challenge it occurs after activation of the immune response. This means the organism is capable of mounting an immune response that can then be restrained by glucocorticoids to prevent the immune system over-responding and posing a threat to the organism. Indeed, removal of these steroids by ADX often has fatal results in response to immune stimulation with cytokines (Blalock, 1989).

The role of the HPA axis in regulating the immune system has been demonstrated in animal models of inflammatory disease. Lewis rats are susceptible to inflammatory disease, such as arthritis and this is associated with HPA axis hyporesponsiveness, whereas Fischer rats are resistant to inflammatory disease and demonstrate HPA axis hyperresponsiveness (Sternberg *et al*, 1989a; Sternberg *et al*, 1989b). Treatment of Fischer rats with the GR antagonist, RU486 renders them susceptible to inflammatory disease, while treatment of Lewis rats with low dose dexamethasone significantly diminishes symptoms of inflammatory disease (Sternberg *et al* , 1989a), suggesting a role for glucocorticoids in regulating the immune system. Cells of the immune system are now known also to produce peptide hormones such as CRH, ACTH, TSH, prolactin, LH and GH (for review see (Blalock, 1989)) classically thought of as being exclusive to neuroendocrine systems. The quantity of hormones secreted by leucocytes is small and their action is probably paracrine or autocrine. Glucocorticoids were the first hormones to be recognised as having

immunosuppressive activity, however the other HPA hormones, CRH and ACTH may also act directly on the immune system (Tomaszewska & Przekop, 1997). Immune cells such as phagocytes and macrophages contain both CRH peptide and CRH receptors (Tomaszewska & Przekop, 1997). I.c.v. or i.v. CRH suppresses natural killer cell activity and decreases T cell proliferation (Tomaszewska & Przekop, 1997); furthermore ACTH receptors have been described on lymphocytes and administration of ACTH restrains several immune functions, including antibody formation and inflammation (Tomaszewska & Przekop, 1997).

1.16.2. Effects of Immune Signals on the HPA Axis

Immune signals have a stimulatory effect on the HPA axis. This action is important so that the appropriate metabolic, behavioural and endocrine changes can be made for restoration of homeostasis following infection and inflammation.

1.16.2.1. Cytokines

Following immune system activation, macrophages increase production of cytokines that regulate immunity and inflammation. Cytokines are a large family of polypeptide mediators that act as intercellular messengers within the immune system by modulating the activity of closely adjacent cells (Balkwill & Burke, 1989). Cytokines interact with specific high affinity receptors to regulate the inflammatory response (Gillis, 1991). These major proinflammatory cytokines include interleukins (IL-1α, IL-1β and IL-6) and tumour necrosis factor alpha (TNF-α). Many studies have provided evidence that cytokines are responsible for informing the brain that an immune response is occurring and stimulating the HPA axis, resulting in increased secretion of ACTH and corticosterone.

1.16.2.2. Cytokine Receptors

There are three major families of cytokine receptors (Foxwell *et al*, 1992): the haematopoietic growth factor receptor family (including the IL-6 receptor), the tumour necrosis receptor family (including two TNF receptors; TNF-R55 and TNF-R75) and the immunoglobulin supergene family (including two interleukin-1 receptors, types I and II). The majority of work has focussed on the IL-1 receptor. The type II IL-1 receptor is a receptor decoy that does not elicit a cellular signal (Colotta *et al*, 1993), which can make interpretation of IL-1 binding studies complicated. Nevertheless, studies have demonstrated that in the rat brain, binding of both radiolabelled IL-1α (Farrar *et al*, 1987) and IL-1β (Katsuura *et al*, 1988) is fairly widespread, with a significant signal in the hypothalamus. In agreement with these findings are *in situ* hybridisation studies which have reported the presence of IL-receptor (type I) mRNA in neurones of the hypothalamus (ventro-medial hypothalamus and arcuate nucleus) as well as in the organum vasculosum of the lamina terminalis (OVLT) (Yabuuchi *et al*, 1994). In addition to the expression in neurones, IL-$1R_1$ mRNA is also expressed on the vascular walls and the epithelial cells of the choroid plexus and the ventricles (Yabuuchi *et al* , 1994). IL-1, type I receptors are also present in the external layer of the median eminence (the site of CRH and AVP parvocellular nerve terminals) (Farrar *et al* , 1987) and the anterior pituitary (Grigoriadis & De Souza, 1989), but not in the adrenal gland (Cunningham Jr *et al*, 1992).

Fewer studies have investigated IL-6 and TNF-α receptor expression in the neuroendocrine system. In the rat, IL-6 receptor mRNA is present in the preoptic, dorso-medial and ventro-medial areas of the hypothalamus, the olfactory bulb, hippocampus (Schobitz *et al*, 1992) and the pituitary gland (Ohmichi *et al*, 1992). TNF-α receptor mRNA is expressed in the pituitary of rats but is not apparent in the brain (Wolvers *et al*, 1993).

1.16.2.3. Effects of Cytokines on the HPA Axis

Given the abundance of cytokine receptors in tissues associated with the HPA axis, it is hardly surprising that cytokines have a profound effect on the HPA activity. A plethora of studies have provided unequivocal evidence that peripheral (i.v. or i.p.) administration of either IL-1 agonists (IL-1α or IL-1β), IL-6 and TNF-α increase plasma ACTH and corticosterone levels in laboratory rats (Sapolsky *et al*, 1987; Berkenbosch *et al*, 1987; Rivier *et al*, 1989; Harbuz *et al*, 1992b) and passive immunisation of rodents against IL-1 attenuates the HPA response to the endotoxin lipopolysaccharide (LPS, a macrophage activator which stimulates release of cytokines (Besedovsky & Del Rey, 1992). The mechanisms by which cytokines released in response to immune challenge activate the HPA axis are not fully understood, but appear complex and involve both centrally mediated responses and actions at the level of the pituitary and adrenal and the vagus nerve. A variety of *in vivo* and *in vitro* studies indicate that the marked increases in circulating ACTH and corticosterone initiated by cytokines are triggered by increased hypothalamic drive to the pituitary corticotrophs. Thus, in the rat, peripheral administration of IL-1β increases secretion of CRH into the portal circulation and this is accompanied by decreased immunoreactive CRH content in the median eminence and consequently increases in plasma ACTH and corticosterone (Sapolsky *et al* , 1987), for review see (Turnbull & Rivier, 1995; Buckingham *et al*, 1996). It appears that CRH is necessary for this effect of IL-1β since immunoneutralisation of endogenous CRH (Berkenbosch *et al* , 1987; Sapolsky *et al* , 1987), or PVN lesion (Rivest & Rivier, 1991) inhibits activation of the HPA axis by peripheral injection of IL-1β. In addition, i.v. IL-1β stimulates CRH mRNA expression in the PVN (Suda *et al*, 1990).

There is conflicting evidence for a concomitant rise in AVP release with CRH following cytokine administration. Earlier studies suggested that cytokines stimulate the CRH neurones in the PVN which are deficient in AVP (Berkenbosch *et al* , 1987), however more recent *in vivo* push-pull perfusion experiments show significant

AVP release at the median eminence (presumably of parvocellular origin), following local hypothalamic administration of IL-1β (Watanobe & Takebe, 1993). Furthermore, central (but not systemic) IL-1β administration increases AVP mRNA expression in the pPVN and triggers increases in ACTH secretion, which are attenuated by administration of AVP antisera (Turnbull & Rivier, 1995).

These data provide convincing evidence that cytokine-induced ACTH secretion is mediated by CRH, however they do not provide definitive evidence for a direct action of cytokines on the hypothalamic PVN neurones. It was therefore necessary to establish whether cytokines activate nerve terminals in the median eminence or whether they stimulate CRH perikarya in the PVN. Studies providing evidence for a dose-dependent increase in ACTH secretion following central administration of IL-1 (α or β) and IL-6 in rats (Katsuura *et al* , 1988; Rivier *et al* , 1989) raised the possibility of different mechanisms of cytokine-induced HPA activation depending on the route of administration. For some time it was thought likely that centrally administered IL-1 acted upon cell bodies of hypophysiotropic neurones within the PVN and systemic IL-1 acted at the level of the nerve terminals of these neurones in the median eminence. Indeed IL-1 injected directly into the median eminence rapidly stimulates ACTH secretion in the rat (Matta *et al*, 1990). This argument was fuelled by the fact that cytokines are large polypeptides (17-26 kDa) and therefore unlikely to cross the blood-brain-barrier (BBB) freely and gain access to targets in the CNS. However, *in vitro* studies have demonstrated the ability of cytokines (IL-1α, IL-1β, IL-6 and TNF-α) to initiate release of CRH from isolated hypothalami (without median eminence) (Navarra *et al*, 1991; Loxley *et al*, 1993) and that IL-6 has fails to evoke CRH release from the isolated median eminence incubated *in vitro* under the same conditions (Navarra *et al* , 1991). Furthermore IL-1 does not stimulate ACTH release from rat anterior pituitary cell cultures (Dunn, 1992), suggesting a direct action of cytokines on CRH secreting cells in the hypothalamus. Moreover, i.c.v. injection of an IL-1 receptor antagonist effectively blocks the HPA response to systemic IL-1β (Fukata *et al*, 1989).

Effects of Cytokines on the Pituitary

The majority of the literature concerning the effects of cytokines on pituitary ACTH secretion argues against a direct role for cytokines in regulating pituitary ACTH release. However, some studies have shown that incubation with IL-1 (α or β), IL-6 or TNF-α (Fukata *et al* , 1989; Ray & Melmed, 1997; Katahira *et al*, 1998) increases ACTH secretion from the mouse corticotrope tumour cell line, AtT-20. Nevertheless, whether this cell line accurately reflects the response of normal pituitary cells to cytokines has been disputed, as inconsistent effects have been reported. Furthermore, long incubation periods (2-8 hours vs 20 min for CRH release) are required if these cytokines are to stimulate ACTH secretion from pituitary cells. Furthermore, IL-1β (Tsagarakis *et al*, 1989a), IL-6 (Navarra *et al* , 1991) and TNF-α (Bernardini *et al*, 1990) have all been shown to evoke CRH release from *in vitro* hypothalamic preparations at concentrations of much lower magnitude than those necessary to elicit ACTH secretion from cultured pituitary cells. Therefore, it seems that rapid elevations of ACTH secretion *in vivo* in response to low doses of cytokines are mediated by increased CRH release and perhaps during conditions of prolonged exposure, cytokines may influence pituitary secretion directly.

Effects of Cytokines on the Adrenal Gland

Despite unsuccessful attempts to detect receptors for IL-1 within the adrenal gland, direct actions of IL-1 on glucocorticoid secretion have been reported. *In vivo* studies have demonstrated a stimulatory effect of IL-1 (given peripherally) on corticosterone secretion when administered to anaesthetised rats with isolated adrenals perfused *in situ* (Roh *et al*, 1987). Similarly, *in vitro* experiments have shown IL-1 (α and β) increases glucocorticoid secretion from quartered rat adrenals (Gwosdow *et al*, 1992), rat adrenal slices (Andreis *et al*, 1991) and from isolated rat (Gwosdow *et al* , 1992) and human (Whitcomb *et al*, 1988) adrenal cells. Similarly IL-6 and TNF-α (Darling *et al*, 1989; Tominaga *et al*, 1991) appear to evoke corticosterone release from adrenal cell cultures, although only receptors for IL-6, and not IL-1 or TNF-α, have been reported in the adrenal gland (Ehrhart-Bornstein *et al*, 1998). However, once again the effects of cytokines on adrenal corticosterone require extremely high

doses and long incubation periods, suggesting that the direct influence of cytokines on adrenal glucocorticoid secretion occurs only after prolonged exposure and therefore may serve to prolong glucocorticoid responses to immune challenge.

1.16.3. How do Cytokines Gain Access to the Brain?

So far the evidence strongly implicates the brain as the primary site at which cytokines exert their effect on the HPA axis. However this poses a problem: how do cytokines gain access to the brain? As mentioned earlier, cytokines are large molecules and therefore are not thought to be able to freely cross the BBB, nevertheless several hypotheses of how cytokines may penetrate the brain have been suggested.

(1) Cytokines may enter the brain at circumventricular organs e.g. organum vasculosum of the lamina terminalis (OVLT) and median eminence (ME).

(2) Active transport of circulating cytokines across the BBB or perhaps cytokines can cross the choroid plexus.

(3) During inflammation the BBB may become more penetrable by cytokines.

(4) An increase in peripheral cytokines may stimulate synthesis and release of central cytokines.

(5) Secondary messengers (released in response to increased circulating cytokines) may activate CRH neurones or excitatory neurones impinging upon them.

While all of these mechanisms may be involved in permitting cytokines access to the brain a growing body of evidence suggests that activation of pPVN neurones need not depend on cytokines entering the brain, but instead their effects are largely mediated by soluble mediators released in response to the actions of cytokines on brain blood vessels. It is thought that once released, these mediators may target the pPVN directly and/or drive the HPA axis by activating neuronal inputs to the PVN. Another possibility is that cytokines activate brain pathways via an action on the vagus nerve (see section 1.16.5.2.).

1.16.4. Eicosanoids

Eicosanoids are lipid mediators derived from arachidonic acid, which are known to contribute to the hypothalamic mechanisms controlling ACTH release (Cowell & Buckingham, 1989; Buckingham *et al* , 1996). Prostaglandins (PGs) are a class of eicosanoids, and their production is stimulated in the presence of elevated circulating interleukins (Dinarello, 1988). A strong body of evidence suggests that IL-1 acts on the endothelium of blood vessels to activate the release of PGs. Indeed brain microvessels express the IL-1 type I receptor (Ericsson *et al*, 1995). PGE_2 action has been implicated during immune challenge (Rivest *et al*, 2000). IL-1 increases release of PGE_2 from rat hypothalamic explants *in vitro* (Navarra *et al*, 1992) as well as from the mPOA, OVLT, PVN and dorsal hippocampus *in vivo* (Komaki *et al*, 1992). Moreover, local injection of PGE_2 into the third ventricle, PVN, OVLT, ME or POA causes a significant increase in HPA activity, reflected by increases in CRH mRNA expression in the PVN and increased ACTH secretion (Katsuura *et al*, 1990; Lacroix *et al*, 1996). Furthermore, both PGE_2 and $PGF_{2\alpha}$ stimulate CRH release from isolated hypothalami (Mulla & Buckingham, 1999). The ACTH response to centrally administered PGE_2 *in vivo* is attenuated by pretreatment with CRH antisera (Cowell & Buckingham, 1989), indicating that CRH is necessary for PGE_2-induced HPA activation. The findings above led to the concept that PGs may be important mediators of the hypothalamic response to immune challenge. Several experimental approaches have been taken to investigate this theory. *In vitro* experiments have shown that indomethacin, a non-selective cyclo-oxygenase (COX; the enzyme complex that forms the common precursor substrate for PG synthesis) inhibitor blocks CRH release by isolated hypothalamic fragments in response to interleukins (Navarra *et al* , 1991). Similarly the importance of eicosanoid pathways has also been reported *in vivo*. Acute treatment with indomethacin (i.v. or i.c.v.) or i.v. ibuprofen (another COX inhibitor) virtually abolishes the effect of IL on ACTH secretion (Katsuura *et al* , 1988; Watanabe *et al*, 1990; Morimoto *et al*, 1991; Rivier, 1993). These COX inhibitors have also been reported to inhibit *c-fos* expression in pPVN neurones induced by i.c.v. and i.p. IL-1β, i.v. IL-6 and endotoxin (Niimi *et al*, 1996; Niimi *et al*, 1997), and to block CRH and AVP release evoked by IL-1β, IL-6

and TNF-α *in vitro* (Navarra *et al* , 1991). Comparable results have been demonstrated employing antibodies against PGE_1, PGE_2 and $PGF_{2\alpha}$. These antibodies prevent IL-1β-induced ACTH secretion (Watanobe *et al*, 1995). Further evidence for a role for PGs in mediating the effects of interleukins on the HPA axis, came from studies showing that the time course of eicosanoid generation in the hypothalamus complements the activation of the pPVN neurones. For example, IL-1β and IL-6 increase the release of PGE_2 from rat hypothalami *in vitro* and *in vivo* along a time course that closely parallels the release of CRH/AVP (Navarra *et al* , 1992).

The site of PG synthesis appears to be within vascular regions of the brain, as indicated by the robust increase in expression of COX-2 mRNA within microvessels in response to systemic inflammation (Cao *et al*, 1996; Cao *et al*, 1998; Lacroix & Rivest, 1998). Moreover, administration of i.v. IL-1β causes a significant rise in COX-2 mRNA expression within endothelial cells of the CNS blood vessels (Lacroix *et al* , 1996).

The actions of PGE_2 are mediated by their specific seven transmembrane receptors, of which there are four subtypes: EP_1, EP_2, EP_3 and EP_4. Three isoforms of EP_3 receptors have been identified, $EP_{3\alpha}$, $EP_{3\beta}$ and $EP_{3\gamma}$ (Rivest *et al* , 2000). *In situ* hybridisation studies have shown EP_3 receptor mRNA and binding sites in neurones of numerous regions of the rat brain, including the ventral septal area (VSA), NTS, VLM and numerous other hypothalamic nuclei (Matsumura *et al*, 1990; Matsumura *et al*, 1992). The EP_2 receptor has been detected in the BNST, lateral septum, subfornical organ (SFO), ventro-medial hypothalamic nucleus, CeA, locus coeruleus and the anterior pituitary; whereas EP_4 receptor mRNA is present in the ventral septum, POA, PVN (magnocellular division), SON, PBN, LC, NTS and VLM (Zhang & Rivest, 1999). The EP_4 receptor seems to play an important role in mediating the HPA response to immune challenge, since administration of LPS, IL-1β (i.v.) or turpentine (i.m.) up-regulates EP_4 mRNA within CRH neurones of the pPVN (within 3h of LPS and IL-1β injection and 6h of the turpentine injection). Activation of the EP_4 containing CRH neurones by these stimuli is reflected by

induction of Fos protein in these cells. The EP_4 transcript is also present in activated catecholaminergic neurones of the NTS, VLM and LC (Zhang & Rivest, 1999) following immune challenge. Together these data support the view that the effects of cytokines on HPA axis activation are mediated via eicosanoids which interact with specific receptors on brain blood vessels to stimulate the release of soluble PGs which can subsequently trigger HPA activation.

1.16.5. Cytokine Signalling

1.16.5.1.Catecholamine Pathways

Central catecholaminergic modulation of the secretion of CRH and ACTH has been well documented (Rivier & Vale, 1983b; Tilders *et al*, 1985; Szafarczyk *et al*, 1985; Guillaume *et al*, 1987) and several lines of investigation have indicated a role for catecholaminergic pathways in driving HPA responses to immune challenge. Systemic IL-1 administration stimulates central and peripheral release of catecholamines, concomitant with an increase in ACTH and corticosterone secretion. Immunocytochemical and *in situ* hybridisation studies have shown induction of *c-fos* mRNA and Fos expression in brain areas such as the NTS and VLM (Day & Akil, 1996), which represent sources of afferent catecholaminergic projections to the PVN, following systemic administration of IL-1β. Several studies have focussed on the effects of interrupting catecholaminergic innervation from the brainstem to the hypothalamus. When such lesions are performed bilaterally, they result in a significant attenuation of the ACTH secretory response and block increases in PVN CRH mRNA expression, following i.v. and i.c.v. IL-1β (Melik Parsadaniantz *et al* , 1995). Moreover, unilateral lesions prevent the induction of CRH mRNA by IL-1β only in the ipsilateral PVN and not the contralateral PVN (Li *et al* , 1996). However, bilateral lesions of ascending catecholaminergic tracts fail to have any effect on ACTH secretion when IL-1β is infused directly into the PVN (Ericsson *et al* , 1994). Administration of adrenoreceptor antagonists also attenuates ACTH secretion and prevents increases in expression of Fos protein in the PVN induced by systemic IL-1β (Givalois *et al*, 1995). The evidence indicates that the actions of IL-1 on HPA

axis activation may in part be mediated by catecholaminergic inputs to the PVN. The mechanisms of how IL-1 activates medullary catecholaminergic neurones are not clear. It is unlikely that IL-1 acts directly on brainstem catecholamine neurones, since there are few IL receptors in this region (Rivest *et al* , 2000). Two alternative mechanisms have been proposed: IL-1β may act on local perivascular cells to trigger the release of PGs, as discussed above or interleukins may act via the vagus nerve (see below). The EP-3 receptor is expressed in the NTS and VLM (Rivest *et al* , 2000) and injection of PGE_2 into the VLM induces patterns of Fos protein expression in the pPVN, similar to those induced by i.v. injection of IL-1β (Ericsson *et al*, 1997). Furthermore, treatment with indomethacin (PG synthesis inhibitor) inhibits the rise in hypothalamic noradrenaline turnover and Fos expression in the pPVN and brainstem (Ericsson *et al* , 1997; Buller *et al*, 1998) normally observed following peripheral IL-1β administration.

1.16.5.2. Vagal Inputs

Cytokines such as interleukins may exert effects within the periphery that influence HPA activity. Several studies have indicated a role for vagal afferents in effecting HPA responses to i.p. endotoxin, IL-1β and TNF-α (Gaykema *et al*, 1995; Fleshner *et al*, 1997; Hansen *et al*, 1998). Vagal lesions inhibit ACTH responses to these cytokines and also block the increases in *c-fos* mRNA expression in the pPVN and NTS, noradrenaline release in the PVN and IL-1β mRNA in the hypothalamus and hippocampus, normally evoked in response to i.p. injection of IL-1β or endotoxin. However, vagotomy does not affect neuroendocrine responses to cytokines administered by other routes, for example, i.v. administration of IL-1β causes increases in CRH mRNA in the pPVN and Fos expression in the pPVN and brainstem, none of which are unaffected by sub-diaphragmatic vagotomy (Ericsson *et al* , 1997). Again, the mechanism by which i.p. cytokines activate the vagal afferents is unclear. These neurones do not seem to express IL-1 receptors (Turnbull & Rivier, 1995), however ganglionic cells which synapse onto vagal afferents are reported to express IL-1 receptors (Mulla & Buckingham, 1999), thus this action may be important. On the other hand, PGs released by macrophages in the

peritoneum may be of primary importance in activation of the vagus (Mulla & Buckingham, 1999). It appears from the data that vagal afferents may provide an important communication link between the immune system and the brain following cytokine administration via the i.p. route.

1.16.6. Cytokine Production

It is known that the brain, pituitary and adrenal gland are all capable of synthesising and secreting various cytokines upon endotoxin stimulation (Turnbull & Rivier, 1995), which suggests cytokines produced in such tissues may regulate HPA activity in a paracrine fashion.

(i) Cytokine Synthesis in the Brain

Expression of IL-1β mRNA (Ban *et al*, 1992) and protein (Hagan *et al*, 1993) have been reported to increase in rat brain (including in the hypothalamus) following systemic administration of endotoxin. Furthermore increased hypothalamic expression of IL-1α, IL-1β, IL-6 and TNF-α mRNA has also been reported following i.p. or i.v. administration of endotoxin in mice (Pitossi F, 1997). The extent to which brain-derived cytokines contribute to hypothalamic responses to immune challenge is not clear. I.c.v. administered IL-1 receptor antagonist significantly suppresses the ACTH response to i.c.v. endotoxin and i.c.v. IL-1β, but does not affect ACTH secretion induced by either i.v. IL-1β or endotoxin (Habu *et al*, 1998). However Kakucska *et al* observed that i.c.v. administration of IL-1 receptor antagonist prevented the rise in plasma ACTH and CRH mRNA expression in the PVN normally observed in rats treated with i.p. endotoxin (Kakucska *et al*, 1993). Hence, endogenous brain cytokines may play a role in mediating HPA activation under certain circumstances and may act to synergise with noradrenaline (released in the hypothalamus following activation of brainstem catecholamine cell groups) to amplify the hypothalamic response to immune insults.

(ii) Cytokine Synthesis in the Pituitary

The pituitary gland is also able to synthesise cytokines. Messenger RNAs for IL-1β (Koenig *et al*, 1990), IL-6 (Muramami *et al*, 1993) and TNFα (Gatti & Bartfai, 1993) have all been demonstrated in the anterior pituitary following peripheral administration of endotoxin.

(iii) Cytokine Synthesis in the Adrenal Gland

Within the adrenal gland, IL-1β immunoreactivity is present in chromaffin cells (Schultzberg *et al*, 1989) and a resident macrophage population (at the cortico-medullary junction) synthesises IL-1, IL-6 and TNF-α. This evidence has suggested a role for these cytokines in stimulating corticosterone secretion following immune challenge (Ehrhart-Bornstein *et al* , 1998). The expression of mRNAs for these cytokines is increased in the adrenal gland within 2 hours of endotoxin administration (i.p. or i.v.) (Ehrhart-Bornstein *et al* , 1998). However, once again the effects of cytokines on adrenal corticosterone secretion require extremely high doses and long incubation periods, suggesting that the direct influence of cytokines on adrenal glucocorticoid secretion occurs only after prolonged exposure and therefore may serve to prolong glucocorticoid responses to immune challenge.

The functional significance of pituitary and adrenal cytokine generation is not well defined. It is possible that cytokines synthesised in the pituitary and adrenal following immune challenge act via local receptors in a paracrine manner to influence ACTH and corticosterone secretion, respectively.

(iv) Cytokine Generation in Response to Non-Immunological Stimuli

In all the cases discussed so far cytokine production has been associated with activation of the immune system, yet it is now generally accepted that other stressors are capable of provoking cytokine production. In the rat, exposure to open-field (LeMay *et al*, 1990), electric footshock (Turnbull & Rivier, 1995) or physical restraint (Zhou *et al*, 1993) results in significantly elevated plasma levels of IL-6, and immobilisation stress stimulates increases in hypothalamic IL-1β mRNA (Minami *et*

al, 1991) and bioactive IL-1 (Shintani *et al*, 1995). Hence, the brain is capable of synthesising interleukins in response to non-inflammatory/infectious stimuli.

In summary, cytokines are released by macrophage cells in response to immune challenge. Cytokines activate the HPA axis, resulting in increased circulating corticosterone. Corticosterone has suppressive actions on the immune response, an effect necessary for maintenance of homeostasis. The brain seems to be the primary site via which cytokines activate hypothalamic CRH/AVP neurones, however due to their large molecular size several mechanisms by which their effects are mediated have been suggested, including via prostaglandins. Prostaglandins may act on receptors to activate brainstem catecholaminergic neurones that project directly to the PVN. Alternatively prostaglandins may act directly on pPVN neurones. Following i.p. cytokine administration, vagal afferents to the brainstem may be important in mediating the influence of cytokines on the HPA axis. The brain, pituitary and adrenal are all well known to be capable of synthesising cytokines. Brain cytokines may have synergistic actions in activating hypothalamic pPVN neurones. Cytokines may also act within the brain, pituitary or adrenal in a paracrine fashion. Finally, evidence suggests that cytokines may be involved in HPA axis regulation in response to non-infectious/non-inflammatory stressors.

1.17. Neuroendocrine Adaptations to Stress in Pregnancy

Virgin animals display complex neuroendocrine responses to stress including activation of the HPA axis and the hypothalamo–neurohypophysial system (HNS) which result in increased circulating ACTH, corticosterone, oxytocin, prolactin, adrenaline and noradrenaline. It is well established that during lactation these neuroendocrine responses to stressful stimuli are altered considerably. The stress–related activation of the HPA axis and HNS system is markedly attenuated, thus less ACTH and corticosterone are secreted by the anterior pituitary and adrenal gland, respectively and less oxytocin is released by the posterior pituitary (Lightman & Young, 1989; Windle *et al*, 1997). More recently, studies have indicated that the

alterations in HPA reactivity to stress, evident in lactation, are established during late pregnancy (Douglas *et al*, 1996; Neumann *et al*, 1998) (da Costa *et al*, 1996).

1.17.1. Basal Activity of the HPA axis in Pregnancy

(i) PVN: CRH and AVP Expression

A recent *in situ* hybridisation study has demonstrated that CRH mRNA expression in the medial parvocellular region of the PVN is significantly reduced (by ~30%) on day 21 of pregnancy (Johnstone *et al*, 2000a). Although the values did not reach statistical significance, AVP mRNA is also apparently decreased since the ratio of AVP mRNA to CRH mRNA content does not change in pregnancy. These data suggest that in late pregnancy, pPVN neurones are synthesising less CRH peptide, however AVP peptide production is not increased to compensate. Compared with CRH, AVP is a less effective secretagogue in triggering ACTH secretion, thus in pregnancy it seems that the factors released by pPVN neurones provide less drive to the anterior pituitary. The reduction in CRH biosynthesis in pregnancy (reflected by reduced CRH mRNA content in the PVN) may be a result of attenuated stimulatory inputs to the PVN neurones, for example from brainstem catecholaminergic afferents (Pacak *et al*, 1996), or as a consequence of increased inhibitory inputs to the PVN, perhaps by GABA neurones (Herman *et al* , 1996), or be a result of enhanced negative feedback by glucocorticoids (Dallman *et al* , 1972).

(ii) ACTH and Corticosterone

In the rat, the circadian variation in ACTH secretion is evident early in pregnancy, but diminishes after mid-gestation (Atkinson & Waddell, 1995). This appears to be due to a reduction in evening peak levels (46 ± 5 pg/ml in diestrus, compared with 25 ± 2 pg/ml on day 22 of pregnancy) (Atkinson & Waddell, 1995). The circadian variation in corticosterone is maintained throughout rat pregnancy, however the absolute levels of plasma corticosterone change. The daily mean (or mesor) declines in early pregnancy, reaching a minimum by day 10 of gestation, but then increases progressively to term (Atkinson & Waddell, 1995). The increases in corticosterone secretion in the second half of pregnancy appears to be as a result of increases in

trough levels with no changes in peak levels and occur without any changes in ACTH. Several factors have been suggested to contribute to the change in the plasma ACTH-corticosterone relationship in pregnancy, including reduced metabolic clearance of corticosterone, foetal corticosterone entering the mother's circulation (indeed surgical adrenalectomy does not abolish maternal circulating corticosterone) or an increased sensitivity of the maternal adrenal gland to ACTH.

A reduction in metabolic clearance of corticosterone can be ruled out since Waddell and Atkinson have shown that corticosterone clearance is maintained at pre-pregnancy levels throughout gestation (Waddell & Atkinson, 1994). However, it is possible that the amount of free corticosterone declines in pregnancy, consistent with an increase in circulating corticosterone binding globulin (CBG) in pregnancy (Lohrenz *et al*, 1967). It seems unlikely that contribution to the maternal pool of corticosterone by the foetal adrenals is the only factor involved in the increases in corticosterone secretion in the second half of pregnancy, since the foetal adrenal only develops the capacity for corticosterone synthesis from day 17 of pregnancy (Milkovic *et al*, 1973), whereas maternal corticosterone levels are increased by day 14. Furthermore, placental 11β-hydroxy-steroid dehydrogenase (11β-HSD; see section 1.20.) should limit the transfer of corticosterone from the foetal to the maternal circulation (Burton & Waddell, 1994). On the other hand, increased responsiveness of the maternal adrenal to ACTH could certainly account for the changes observed in the ACTH-corticosterone relationship in pregnancy. Oestrogen is known to increase the sensitivity of the adrenal to ACTH (Carr *et al*, 1981) perhaps by increasing ACTH receptors, and secretion of oestrogen from the ovary increases during the second half of pregnancy in the rat (Schulte *et al*, 1990; Waddell & Atkinson, 1994).

1.17.2. HPA Axis Responses to Stress in Pregnancy

The responsiveness of the HPA axis to both emotional (elevated plus maze) and a combined emotional-physical stressor (forced swimming) is significantly attenuated in pregnancy, reflected by reduced stress-induced ACTH and corticosterone

secretion (Neumann *et al* , 1998). The hyporesponsiveness of the HPA axis to stress is evident from day 15 (Neumann *et al* , 1998) of gestation and persists through pregnancy and lactation, until weaning (Windle *et al* , 1997). The attenuated responsiveness of the HPA axis in pregnancy is likely to involve adaptations at the level of the anterior pituitary. *In vivo* experiments have demonstrated attenuated ACTH secretory responses (a 4.4-fold increase in virgin rats compared with a 1.5-fold increase in day 21 pregnant rats) to intravenously administered CRH (Neumann *et al* , 1998), indicating reduced reactivity of pituitary corticotrophs to CRH. Similarly, CRH is less effective in stimulating cAMP production from pituitary segments isolated from late pregnant rats (day 17 and day 22) compared with those of virgin animals (Neumann *et al* , 1998). Furthermore, receptor autoradiography has revealed a significant reduction in radio-labelled CRH binding site density in the anterior pituitaries of pregnant rats (on day 11, day 17 and day 22) (Neumann *et al* , 1998).

A more recent study has reported increased 11β-HSD-1 activity in the anterior pituitary in late pregnancy (Johnstone *et al* , 2000a). Since 11β-HSD-1 predominantly acts as a reductase, reactivating glucocorticoids from their inert forms, its increased activity in pregnancy would presumably enhance local glucocorticoid levels, thereby increasing the negative feedback action of corticosterone on anterior pituitary corticotrope cells. In the same study, 11β-HSD activity in the PVN was found to have doubled by day 16 and trebled by day 21 of pregnancy (Johnstone *et al* , 2000a), suggesting a role for enhanced glucocorticoid feedback mechanisms on pPVN CRH neurones as well as anterior pituitary corticotrope cells. Moreover GR mRNA expression in the dentate gyrus was also increased at the end of pregnancy (Johnstone *et al* , 2000a). However, i.c.v. administration of the 11β-HSD inhibitor, glycyrrhetinic acid (GA) was not effective in increasing the ACTH secretory response to forced swimming in the pregnant group (Johnstone *et al* , 2000a). Furthermore, removal of endogenous corticosterone by pharmacological ADX (phADX), results in a comparable elevation of plasma ACTH levels in both virgin and pregnant rats, indicating that endogenous glucocorticoids restrain ACTH secretion similarly in both groups (Johnstone *et al* ,

2000a). Pregnant rats also appear to be less sensitive to rapid glucocorticoid feedback as demonstrated by ACTH responses in phADX rats to s.c. corticosterone administration, but are similarly sensitive to delayed corticosterone feedback (Johnstone *et al* , 2000a). These data suggest that reduced neural drive to PVN CRH/AVP neurones may account for the hyporesponsiveness of the HPA axis to stress in pregnancy, rather than enhanced negative feedback by glucocorticoids. Supporting this theory is a study demonstrating that *c-fos* mRNA expression in the pPVN of late pregnant rats (day 19-21) is significantly reduced following 30 minutes of restraint, compared with the respective virgin group (da Costa *et al* , 1996).

1.17.3. Oxytocin Secretory Responses to Stress in Pregnancy

As mentioned earlier (see section 1.11), exposure to stressors stimulates oxytocin secretion in the rat. While oxytocin may act to stimulate corticosterone secretion from the adrenal gland, its functional significance in coping with stress is not clear. In lactating rats, the oxytocin secretory response to stress is markedly attenuated (Lightman & Young, 1989). This may be a consequence of depleted neurohypophysial oxytocin stores due to the frequent pulses of oxytocin release required for the milk-ejection reflex (Higuchi *et al*, 1991). During pregnancy, in preparation for parturition, the oxytocin content of the rat posterior pituitary increases progressively (Douglas *et al* , 1993; Douglas *et al* , 1995) and the accumulated store is secreted during parturition to stimulate uterine contractions and foetal expulsion. The build up of oxytocin stores occurs as a result of active restraint of oxytocin secretion by endogenous opioids (Douglas *et al* , 1993; Douglas *et al* , 1995). However, in contrast to lactation, pregnant and virgin rats demonstrate similar oxytocin secretory responses to forced swimming (Douglas *et al*, 1998). Moreover, oxytocin secretory responses to forced swimming in pregnancy are strongly enhanced by naloxone (an opioid antagonist), which indicates that endogenous opioids actually mask an exaggerated response after exposure to stress. Previous studies have shown that endogenous μ-opioids strongly inhibit oxytocin neuronal activity and secretion in late pregnancy, at the level of oxytocin cell bodies and their inputs (Douglas *et al* , 1995). The opioid restraint on oxytocin secretion following

stress in pregnancy may involve a similar mechanism, though action on the inputs to oxytocin neurones is more probable since endogenous opioids do not appear to be responsible for attenuated oxytocin secretory responses to hyperosmotic stimulation (Bull & Russell, 1992) or electrical stimulation of the OVLT (Bull *et al*, 1994) in pregnancy.

1.17.4. Opioids and the HPA Axis in Pregnancy

Opioids are known to play an important modulatory role in regulating both HPA activity and oxytocin secretion in response to stress (see section 1.16). Since in late pregnancy (from day 16 onwards) endogenous opioids exert inhibitory tone on oxytocin neurones (Douglas *et al* , 1993; Douglas *et al* , 1995), it would seem plausible that a similar central inhibitory opioid mechanism may be involved in the attenuated HPA responses to stress stimuli in pregnancy. Pretreatment with naloxone significantly attenuates the rise in ACTH secretion in virgins exposed to forced swimming, while pregnant rats demonstrate a modest increase in ACTH secretion (Douglas *et al* , 1998). It would seem that pregnant rats no longer display the opioid enhancing effects on ACTH secretion observed in virgins, and instead endogenous opioids have a net inhibitory action over HPA activity in pregnancy. The site at which endogenous opioids may act to restrain HPA responses to stress in pregnancy and their source is unclear, however it is possible that arcuate nucleus POMC neurones which project to the PVN may play a role, as may co-produced opioids in brainstem neurones projecting to the PVN (see Chapter 4).

1.17.5. Sex Steroids and Neuroendocrine Responses to Stress in Pregnancy

A clear sex difference in stress-induced secretion of pituitary hormones exists. Naloxone significantly potentiates the oxytocin secretory response to immobilsation stress in female rats, but not in males (Carter *et al* , 1986) and this is partly determined by gonadal hormones, since testosterone acting in the preoptic area has inhibitory actions.

Since there is greatly increased production of the sex steroids, oestrogen and progesterone in pregnancy, it would seem likely that they may be involved in the physiological adaptations of the neuroendocrine system which occur at this time. In the rat, oestrogen and progesterone levels peak in mid-gestation, around the time when these neuroendocrine adaptations are first observed; followed by progesterone withdrawal 1-2 days before parturition (on day 22) (Bridges, 1984). Treatment of virgin rats with s.c. oestradiol and progesterone capsules for 17 days strongly enhances the oxytocin secretory response to forced swimming in the presence of naloxone (Douglas *et al*, 2000). This indicates that oestrogen and progesterone treatment not only up-regulates the oxytocin secretory response to forced swimming, but also induces strong opioid inhibition of this response. Thus sex steroids appear to be partly responsible for enhanced oxytocin neurone responsiveness and induction of endogenous opioid restraint of oxytocin secretion in pregnancy. Thus it seems that sex steroids are important in pregnancy for inducing opioid restraint over oxytocin secretion and enhanced oxytocin neurone responsiveness.

There is little evidence to support a role for sex steroids in the hyporesponsiveness of the HPA axis in pregnancy. Indeed virgin rats implanted subcutaneously with 17β-oestradiol and progesterone capsules (for 17 days) to mimic pregnancy levels of circulating sex steroids do not demonstrate attenuated ACTH or corticosterone secretory responses to forced swim stress (Douglas *et al* , 2000).

The significance of a hyporesponsive HPA system in pregnancy may be that it provides a protective mechanism for the foetuses. Restraining the maternal HPA axis may avoid overwhelming the placental mechanism which limits the passage of corticosterone across the placenta (see section 1.20.3.), thus protecting the offspring from the detrimental consequences of exposure to excessive levels of corticosterone *in utero* (see section 1.21.). There may also be metabolic advantages to the pregnant mother (see Chapters 5 and 8)

1.18. Placental CRH and POMC

The presence of CRH in human placental extracts (closely related to CRH of hypothalamic origin) was reported by Shibasaki and colleagues in 1982 (Shibasaki *et al*, 1982). In humans, CRH of placental origin is the main source of circulating CRH in pregnancy. Placental CRH output dramatically increases as pregnancy progresses in humans and is accompanied by increased CRH gene expression in the placenta (McLean & Smith, 1999). Production of placental CRH appears to be species-specific, since although CRH activity has also been detected in the sheep (Jones *et al*, 1989), neither CRH protein nor mRNA has been detected in rat or guinea pig placentae (Robinson *et al*, 1989).

In sheep and humans maturation of the fetal HPA axis is important for the stimulus of the onset of parturition (for review see Riley & Challis, 1991). Although glucocorticoids exert negative feedback on PVN CRH neurones, they have a positive effect on CRH release from amnion, chorion and decidual placental cells. Local CRH effects on human and sheep placenta, decidua and myometrium include stimulatory roles on local production of oxytocin and prostaglandins, blood vessel dilation and uterine contractility (Reis *et al* , 1999). CRH released from the foetal membranes and placenta provides further stimulation to the fetal HPA (and possibly the maternal HPA) axis, thus establishing a feedforward loop, the outcome of which is parturition.

The presence of POMC mRNA in rat placenta has been reported, however expression is very low and does not change throughout the duration of pregnancy (Chen *et al*, 1986). Since placental CRH is undetectable and POMC expression is constitutively low throughout pregnancy in the rat, it is unlikely that they are involved in regulation of parturition in this species. However this also means that the rat is a good model in which to study the adaptations of the HPA axis in pregnancy, since there are no unnecessary complications with respect to the impact of placental CRH and/or POMC on maternal HPA function.

1.19. CRH-Binding Protein

In humans, the liver secretes CRH binding protein (CRH-BP) which protects the corticotrophs in the anterior pituitary from domination by placental CRH. The existence of a binding protein for CRH was first reported by Linton *et al* in 1986 (Linton & Lowry, 1986) who demonstrated its presence in human plasma. Although CRH concentrations in the peripheral circulation are normally low, they increase throughout pregnancy in humans (Suda *et al*, 1985; Linton *et al*, 1987), and in the third trimester reach levels which when present in hypophyseal portal plasma elicit ACTH release (Plotsky & Sawchenko, 1987). Nevertheless, maternal plasma ACTH concentrations remain within non-pregnant limits throughout gestation (Rees *et al*, 1975).

CRH binding protein is a 37-kDa protein capable of binding CRH with high affinity (Linton & Lowry, 1986) and negating its biological activity, thereby preventing inappropriate pituitary-adrenal stimulation in pregnancy. CRH-BP has been partially sequenced and cloned (Potter *et al*, 1991; Behan *et al*, 1993) from both human liver and rat brain libraries. The structures of the human and rat CRH-BPs are highly conserved, showing 85% identity in amino acid sequence, however the pattern of tissue distribution of CRH-BP transcripts in the two species is distinct. In humans the CRH-BP gene is expressed in liver, placenta and brain, whereas in the rat CRH-BP mRNA has only been detected in the brain (Potter *et al* , 1991; Baigen & Lowry, 2000). This may reflect differences in placental CRH production. In primates the placenta also produces CRH, whereas the rodent placenta does not produce significant amounts of CRH and therefore peripheral CRH-BP is not required to antagonise the biological activity of placental CRH and prevent inappropriate effects on the pituitary.

Combined immunohistochemical and *in situ* hybridisation studies have revealed CRH-BP mRNA and protein is expressed throughout the rat CNS, in the cerebral cortex, the amygdaloid complex and the BNST (Potter *et al*, 1992). In the brainstem,

sensory relays associated principally with the auditory, olfactory, trigeminal and vestibular systems and several raphe nuclei of the midbrain and pons, express CRH-BP hybridised and immunoreactive neurones (Potter *et al* , 1992). Instances of CRH-BP and CRH peptide colocalisation have been demonstrated in the BNST, median preoptic area (mPOA), CeA, mesencephalic central gray, interpeduncular nucleus, lateral dorsal tegmental nucleus, inferior colliculus, lateral reticular nucleus and olfactory bulb. Interestingly, evidence suggests potential CRH-CRH-BP interactions. Dense CRH-BP stained nerve terminal fields have been shown to coincide with cell groups rich in CRH immunoreactive perikarya in the BNST, CeA and SCN (Potter *et al* , 1992). Furthermore CRH-BP immunoreactivity and mRNA are expressed extensively and almost exclusively over ACTH-positive cells in the anterior pituitary (Potter *et al* , 1992), and CRH-BP is capable of inhibiting CRH-induced ACTH release from pituitary cultures (Potter *et al* , 1991). Taken together these data imply that CRH-BP, an endogenous modulator of the biological activity of CRH in the periphery, could interact with CRH and/or its receptor to modulate the synaptic and hormonal actions of CRH at select sites in the CNS and pituitary.

1.20. 11β-Hydroxysteroid Dehydrogenase

It is well established that as well as CBG and type II corticosteroid receptors, control of glucocorticoid action is also regulated by pre-receptor enzyme-mediated metabolism. 11β-hydroxysteroid dehydrogenase (11β-HSD) plays an important role in determining the access of glucocorticoids to both MR and GR in peripheral tissues (Seckl & Brown, 1994). 11β-HSD catalyses the interconversion of corticosterone (cortisol in humans) and 11-dehydro-corticosterone (see figure 1.7.). The latter has little intrinsic affinity for MR or GR and is considered inert. There are two distinct isoforms of 11β-HSD. The first, 11β-HSD-1, is a NADP(H)-dependent enzyme with a relatively low affinity for corticosterone (Lakshmi & Monder, 1988; Agarwal *et al*, 1989) which catalyses predominantly the reductase reaction to regenerate active corticosterone (Low *et al*, 1994; Rajan *et al*, 1996). The second, 11β-HSD-2, is a NAD-dependent, exclusive dehydrogenase, which inactivates corticosterone, for which it has very high affinity (Brown *et al*, 1993; Albiston *et al*, 1994). 11β-HSD-1

has fairly widespread distribution with high levels found in the liver, while 11β-HSD-2 is most abundantly expressed in the placenta (Waddell *et al*, 1998). In the placenta 11β-HSD-2 provides a barrier to protect the foetus from maternal glucocorticoid concentrations (Seckl, 1994), which can have damaging effects on the foetus (see section 1.22.).

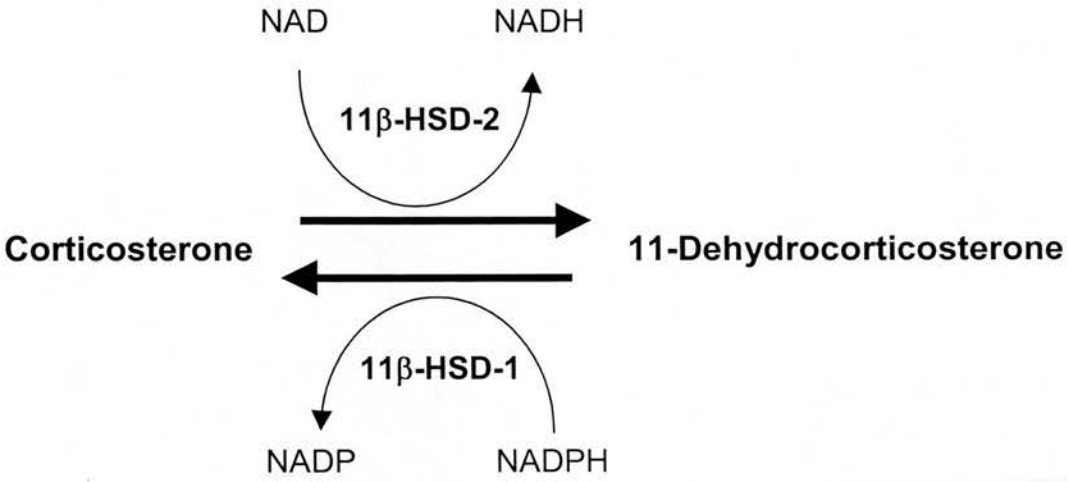


Figure 1.7. Interconversion of corticosterone and 11-dehydrocorticosterone by 11β-HSD. The 11β-HSD type 1 enzyme acts as a reductase to convert 11-dehydrocorticosterone into active corticosterone, whereas the 11β-HSD type 2 enzyme acts as a dehydrogenase and inactivates corticosterone.

More recently immunocytochemical and *in situ* hybridisation studies have shown that 11β-HSD-1 protein and mRNA expression is fairly widespread in the brain. 11β-HSD has been detected in the cerebellum, brainstem, hippocampus (including the dentate gyrus), the neocortex, some hypothalamic nuclei (including the PVN) and most anterior pituitary cells (Moisan *et al*, 1990a; Moisan *et al*, 1990b). Reverse transcriptase-PCR studies have demonstrated 11β-HSD-2 transcripts are present in most brain subregions, including the hippocampus, cerebellum, cortex, brainstem, hypothalamus and pituitary (Seckl, 1997). However *in situ* studies are discordant,

11β-HSD-2 mRNA expression has been reported to have a restricted distribution in the commissural NTS (cNTS) subcommissural organ (SCO), VMH and medial vestibular nucleus (Seckl, 1997). Thus further studies are necessary to confirm the distribution of 11β-HSD in the brain.

1.20.1. Effects of 11β-HSD on the HPA Axis

11β-HSD-1 mRNA and protein are expressed in many magnocellular and parvocellular PVN neurones (Sakai *et al*, 1990). It has been suggested that in the PVN, 11β-HSD-1 is colocalised with MRs and GRs (Sakai *et al* , 1990). Glycyrrhetinic acid (GA) an active component of liquorice, inhibits 11β-HSD activity both *in vitro* (Moisan *et al* , 1990a) and *in vivo* (Monder *et al*, 1989). Treatment of rats with GA causes CRH levels in hypophysial portal blood to fall to ~50% of control values (Seckl *et al*, 1993) suggesting that 11β-HSD regulates the effective corticosterone feedback signal to pPVN CRH neurones. This effect is abolished in ADX rats, suggesting dependence on adrenal products (Seckl *et al* , 1993). In contrast, GA treatment induces two- to threefold increases in arginine vasopressin and oxytocin release into portal blood, effects also dependent upon intact adrenal glands (Seckl *et al* , 1993). These data suggest that 11β-HSD in the PVN, and possibly other sites, may represent an important control point of corticosteroid feedback on CRH release *in vivo*.

1.20.2. Placental 11β-HSD

Glucocorticoids are for the most part prevented from entering the foetal compartment by placental 11β-HSD-2 (Seckl, 1994; Benediktsson *et al*, 1995). However the efficiency of placental exclusion of corticosterone varies considerably in rats and humans (Benediktsson *et al*, 1993a; Benediktsson *et al* , 1995). Nonetheless the presence of this enzyme in placental tissue is of vital importance in regulating foetal environment and may help protect the foetus from adverse programming by corticosterone (see section 1.21.).

1.21. Foetal Programming

Stress is a risk factor for a range of diseases, from auto-immune disorders to mental illness. As mentioned earlier, the typical response to stress involves activation of the HPA axis, resulting in increased secretion of glucocorticoids from the adrenal cortex. The adrenal medulla releases catecholamines, such as adrenaline and noradrenaline in response to stress. These adrenal compounds act together triggering a switch in metabolism, which favours glucose production. Circulating catecholamines activate glycogenolysis and rapidly augment free fatty acid supply to the heart and muscles. Glucocorticoids facilitate this lipolysis and also provide amino acid substrate for gluconeogenesis. Together these hormones shift glucose utilisation towards the CNS and away from peripheral tissues and raise blood pressure and cardiac output, so as to improve delivery of substrates to tissues that are critical to the immediate defence of the organism. Hence the availability of energy is increased to enable the organism to cope with the stress. Obviously prolonged activation of the "stress response" outlined above poses a risk to the health of an individual. Studies have indicated that prolonged or exaggerated stress responses increase susceptibility to steroid-induced diabetes, atherosclerosis and hypertension (Brindley & Rolland, 1989), conditions that are associated with an increased risk of heart disease (Seeman *et al*, 1997). In addition, chronic activation of the HPA axis can affect cognitive function and increase vulnerability to states of anxiety and depression (Arborelius *et al*, 1999). So, a problem arises: the "stress responses" necessary for coping with and surviving stressful events can, if not kept in check, ultimately lead to disease. Substantial evidence indicates that early life events, both pre- and post-natal, can influence the development of the HPA axis and have long term effects on its responsivity to stress. The concept that non-genetic factors act early in life to imprint physiological systems is referred to as "programming".

The majority of studies on the effects of prenatal stress (preNS) have been performed in rodents. Pregnant dams have been exposed to various stressors, e.g. restraint (Deminiere *et al*, 1992), immobilisation (Ward & Weisz, 1984), noise (Fride &

Weinstock, 1984) and electric tail shocks (Takahashi & Kalin, 1991), usually on an unpredictable basis throughout or during a specific period in pregnancy. These studies have provided evidence that maternal stress has permanent and often profound effects on the offspring. Prenatally stressed rats display altered, usually exaggerated neuroendocrine and behavioural responses to stress (Dunn & Berridge, 1990a).

1.21.1. Effects of Prenatal Stress on Behaviour, Anxiety and Cognition

In adulthood prenatally stressed (preNS) rats tend to display a higher degree of anxiety related behaviours (Fisher & Brown, 1991). Studies in rats and monkeys have demonstrated that preNS animals display behavioural abnormalities, including a reduced propensity to play (Takahashi *et al*, 1992) and disturbed social interactions (Schneider, 1992; Schneider & Coe, 1993; Clarke & Schneider, 1993). When preNS rats are exposed to 'emotionality' tests, such as the open field, they typically show reduced locomotion and exploration and increased defecation (Wakshlak & Weinstock, 1990; Poltyrev *et al*, 1996) and they also spend less time on the open arms of the elevated plus maze (EPM) (Poltyrev *et al* , 1996; Vallee *et al*, 1997). Anxiolytic drugs such as benzodiazepines significantly increase the number of entries rats make onto the open arms of the EPM (Pellow & File, 1986) confirming that preNS rats are more anxious. Chronic stress exposure or i.c.v. administration of CRH to rats, induces these behaviours seen in preNS rats, including decreased social interaction (Dunn & File, 1987), suppression of locomotion in the open-field (Britton *et al*, 1982) and aversion to the open arms of the EPM (Dunn & Berridge, 1990a). Together these data show that prenatal stress programmes the brain, leading to increased anxiety-related behaviour in adulthood, an effect possibly mediated via enhanced CRH release.

Retrospective studies on human behaviour have shown that children of mothers who experienced psychological stress during pregnancy (e.g. marital problems, death of spouse, war) exhibit behavioural abnormalities, including excessive clinging, crying, hyperactivity, unsociable and inconsiderate behaviour (Stott, 1973). Another study

has demonstrated that children born to mothers whose spouse died during their pregnancy were more likely to experience psychiatric disorders at adolescence including schizophrenic episodes, minor depressive symptoms, alcoholism and asocial or criminal behaviours (Huttunen & Niskanen, 1978). In the early 1990s a link between prenatal stress and hyperactivity-attentional deficit disorder was suggested (Clements, 1992). The evidence from human studies is in accord with the animal data, supporting the concept that stress during pregnancy can programme for behavioural abnormalities later in life.

Adult cognition is also clearly affected by prenatal stress. PreNS rats spend more time looking for the platform in a watermaze (Szuran *et al*, 1994; Hayashi *et al*, 1998). This may be due to the reduced levels of hippocampal corticosterone receptors (Weinstock *et al*, 1992; Henry *et al*, 1994) since water-maze performance is a hippocampus-associated function, influenced by occupation of corticosterone receptors (Morris *et al*, 1982; Oitzl & De Kloet, 1992; Oitzl *et al*, 1997; Sandi, 1998). Thus exposure to elevated corticosterone levels in preNS rats may lead to hippocampal damage (as observed in aged rats) and explain poorer performance in tests of cognitive function.

1.21.2. Effects of Prenatal Stress on the HPA Axis and its Regulation

In prenatally stressed rats, increased basal HPA activity has been reported. The effects of preNS on circulating ACTH and corticosterone levels are more marked in females (McCormick *et al*, ; Weinstock *et al* , 1992). Some studies have demonstrated elevated corticosterone levels during the pre-weaning period in male rats (Takahashi & Kalin, 1991), whereas others have reported no effect of preNS on basal corticosterone and ACTH secretion in males at this time (Fride *et al*, 1986; Takahashi *et al*, 1990; Henry *et al* , 1994), these differences may reflect the type of stressor used and the period of gestation in which the stress was administered. Nevertheless, stress-induced increases in ACTH and corticosterone are consistently higher in preNS animals (McCormick *et al* , ; Peters, 1982; Fride *et al* , 1986; Takahashi & Kalin, 1991; Weinstock *et al* , 1992) and post-stress corticosterone

levels show prolonged elevation compared with controls (Takahashi & Kalin, 1991; Henry *et al* , 1994). Furthermore preNS rats fail to adapt to repeated homotypic stressors and continue to release high amounts of corticosterone (Fride *et al* , 1986). The mechanisms underlying the impaired feedback regulation of the HPA axis in preNS animals are not completely understood. On reaching adulthood, preNS rats demonstrate higher circulating corticosterone levels, concomitant with reduced levels of hippocampal MR and GR receptors (Henry *et al* , 1994). This evidence implies abnormal negative feedback control of CRH release in preNS rats. On postnatal day 3, preNS rats have higher levels of plasma corticosterone than control pups, but similar numbers of hippocampal MRs and GRs (Henry *et al* , 1994). The reduction in MRs and GRs is not observed until weaning (~ postnatal day 21), but persists into adulthood (Henry *et al* , 1994). Thus increased corticosterone levels precede changes in MR and GR binding and hence elevated HPA activity in neonatal life may be responsible for altered development of hippocampal MR and GR expression.

PreNS offspring have higher levels of CRH in the amygdala (Cratty *et al*, 1995). This may be a consequence of increased levels of plasma corticosterone, since glucocorticoid administration has been shown to increase CRH mRNA in the CeA (Makino *et al*, 1994). It is tempting to speculate that increased amygdala CRH content in preNS rats may underlie their anxiety related behaviour (discussed earlier) especially since CRH micro-injection into the amygdala (Davis, 1992) induces similar 'anxiety' behaviours. PreNS rats also have less brain opioid receptors than controls (Insel *et al*, 1990) and a decrease in POMC mRNA in the hypothalamus (Weinstock *et al* , 1992). β-endorphin has been shown to have an inhibitory action on CRH release (Buckingham, 1986), and blockade of opioid receptors by naloxone (Siegel *et al*, 1982; Odio & Brodish, 1990) increases plasma corticosterone (in control rats), thus a decrease in inhibitory tone by opioids could explain the higher corticosterone levels in preNS rats. However, it is unlikely that reduced opioid mediated inhibition is the sole mechanism responsible for elevated corticosterone levels in preNS rats, since these rats show larger increases in corticosterone levels following treatment with naloxone than control rats do (Weinstock *et al*, 1996).

1.21.3. Effects of Prenatal Stress on Brain Amines

Under basal conditions, preNS rats demonstrate significantly higher noradrenaline turnover in the cerebral cortex and locus coeruleus with similar effects observed following electric foot-shock treatment (Takahashi *et al*, 1992). Furthermore, increases in noradrenaline release in the amygdala and hippocampus may lead to a reduction in corticosterone feedback inhibition of CRH by decreasing the number of glucocorticoid receptors (Maccari *et al*, 1992). States of conditioned fear and anxiety are characterised by increased dopamine turnover in the prefrontal cortex and decreased dopamine turnover in the striatum and nucleus accumbens (Claustre *et al*, 1986). In accord with their augmented anxiety in stressful situations, preNS rats have higher dopamine turnover in the prefrontal cortex and lower turnover in the striatum and nucleus accumbens (Fride & Weinstock, 1988). Changes in dopamine turnover in preNS rats explain the reduction in exploratory activity in behavioural tests of anxiety.

1.21.4. Effects of Prenatal Administration of Glucocorticoids

It seems likely that maternal glucocorticoids play a role in the effects of preNS on the foetus (Barbazanges *et al*, 1996) and many studies have investigated the effects of glucocorticoid administration to pregnant rats on behavioural and HPA function of the offspring. Dexamethasone is a synthetic glucocorticoid and a potent GR (but not MR) agonist, which can readily cross from mother to foetus via the placenta. Prenatal dexamethasone exposure throughout gestation or in the last week only has been shown to induce anxiety-related behaviour in adult rats in the open-field test (Welberg *et al*, 2001; Welberg & Seckl, 2001). These behavioural changes may be associated with amygdalar adaptations, since prenatal dexamethasone treatment (either throughout or in the last trimester) increases CeA expression of CRH mRNA (Welberg *et al* , 2001; Welberg & Seckl, 2001). Dexamethasone administration to rats on days 15-20 of gestation results in reduced pup birth weights (which is strongly associated with hypertension in adulthood). In adulthood, the offspring

display elevated blood pressure (Lindsay *et al*, 1992; Benediktsson *et al*, 1992; Edwards *et al*, 1993; Benediktsson *et al*, 1993b; Levitt *et al*, 1996) and increased basal levels of plasma corticosterone (Levitt *et al* , 1996). Moreover, hippocampal GR and MR mRNA expression are significantly and permanently attenuated (Levitt *et al* , 1996) compared to controls. This may underlie reduced feedback inhibition by glucocorticoids and explain elevated corticosterone levels in preNS rats.

If glucocorticoids are in fact involved in mediating the effects of preNS on the foetus, then 11β-HSD2 may play an important role in modulating the programming effects of prenatal exposure to endogenous glucocorticoids. Although 11-HSD2 in the placenta acts as a barrier to maternal glucocorticoids, this barrier is leaky and it can become saturated (Shibasaki *et al* , 1982). Endogenous inhibitors of 11β-HSD activity have been described (Buhler *et al*, 1994) which may also be involved in regulating foetal exposure to maternal corticosterone. Inhibition of feto-placental 11β-HSD2 with carbenoxolone (CBX) during pregnancy decreases birth weight of the offspring and programmes permanent hypertension and hyperglycaemia in adulthood (Edwards *et al* , 1993; Lindsay *et al*, 1996a; Lindsay *et al*, 1996b; Welberg *et al*, 2000). These effects are abolished if the mother is adrenalectomised (Lindsay *et al* , 1996a; Lindsay *et al* , 1996b), indicating that inhibition of 11β-HSD2 allows increased levels of maternal corticosterone to access foetal GR, hence programming the offspring. CBX treatment also affects HPA activity of the offspring, causing increased basal and stress-induced corticosterone levels and CRH mRNA in the PVN (Welberg *et al* , 2000). GR mRNA expression is reduced in the PVN of prenatally treated CBX rats, suggesting that attenuated corticosterone feedback may be involved in the HPA adaptations. Furthermore anxiety-like behaviours similar to those observed in preNS rats are displayed in adult prenatally CBX treated rats. Thus exposure to elevated glucocorticoids during pregnancy as a result of prenatal stress or feto-placental 11β-HSD dysfunction can permanently programme the behaviour and neuroendocrine responses of the offspring.

1.21.5. Effects of the Post-Natal Environment on the Offspring

The post-natal environment can also influence the activity of the HPA axis and anxiety behaviour (for reviews see (Meaney *et al*, 1994; Ladd *et al*, 2000; Champagne & Meaney, 2001)). Maternal behaviour plays an important role in the development of the HPA axis. In rats, high licking and grooming behaviour directed towards the pups by the mother and arched back nursing have been found to correlate with reduced PVN CRH mRNA expression, enhanced glucocorticoid negative feedback and lower stress responses (ACTH and corticosterone) in adulthood (Champagne & Meaney, 2001). Furthermore, early maternal separation results in exaggerated HPA responses to stress (restraint or novelty) later in life, an effect that can be abolished if the pups are handled and stroked during maternal deprivation (Meaney *et al* , 1994). The mechanisms responsible for this are not known, however they are likely to involve altered glucocorticoid feedback sensitivity (Plotsky & Vale, 1984; Plotsky *et al*, 1986; Sawchenko, 1987; Plotsky & Sawchenko, 1987; Owens *et al*, 1990; Imaki *et al*, 1991a). Since the HPA axis continues to develop after birth in the rat, the possibility that the adverse programming effects of exposure to prenatal stress can be reversed or modulated by the postnatal environment and maternal behaviour is an exciting area which requires more research and may in the future have important implications for humans.

What are the important questions that arise?

Taking into account the literature reviewed above several key questions arise. The ACTH and corticosterone secretory responses to two stressors (namely the elevated plus maze and forced swimming) are markedly attenuated in pregnancy, however what causes these changes are not known.

- Is this a consequence of reduced activation of the pPVN CRH/AVP neurones by stress in pregnancy?
- Does the HPA axis respond less to other emotional stressors in pregnancy, including those that are perhaps more physiologically relevant i.e. responses to social stress?
- Is the HPA axis also less responsive to immune challenge in pregnancy?
- Metabolic signals are known to activate the HPA axis, and clearly metabolism is altered in pregnancy, however no study has investigated the effects these signals have on the HPA axis in pregnancy.
- Are the changes in HPA axis responsiveness in pregnancy a result of the CRH/AVP neurones themselves being less responsive to all excitatory stimuli?
- If so, is this a result of increased inhibitory influences in the pPVN (e.g. by endogenous opioids)?
- Is glucocorticoid feedback inhibition of HPA activity altered in pregnancy to restrain the axis?
- What about other species? Are the changes in HPA axis responsiveness to stress in pregnancy in the rat species-specific or do pregnant mice display similarly reduced responses to stress?

Why is it important to understand the mechanisms involved in reduced HPA axis responses to stress in pregnancy?

Pregnancy is associated with a series of adaptive changes necessary for the development and maintenance of the foetus. These changes are necessary in order that the maternal physiology meets both her demands and those of the foetus. One of

the major adaptations that occurs during pregnancy is the change in responsiveness of the HPA axis. The aim of this thesis is to investigate the brain mechanisms (with particular focus on the hypothalamus) that occur in pregnancy and permit reduced neuroendocrine responses to stress, and will maximise the chances of a successful outcome. The plasticity of the HPA axis in pregnancy is in itself an interesting focus of study, however the need to understand the mechanisms involved relates also to the long term well-being of the foetus and the mental health of the mother. Exposure to prenatal stress has programming effects on the foetus that can have life-long consequences on the neuroendocrine and immune systems as well as on behaviour and cognition. Furthermore, given the well established involvement of CRH in depressive illness (Gutman *et al*, 2001), a rebound in HPA activity from a dampened state in pregnancy may be involved in the manifestation of post-natal depression. Thus understanding the mechanisms underlying reduced stress responses in pregnancy has potential benefits to both the mother and her offspring.

Aims of Thesis

The experiments in this thesis were designed to investigate changes in the central mechanisms underlying attenuated HPA axis responses to stress in late pregnant rats. Several approaches were taken and the specific aims were:

- to investigate whether the HPA axis is less responsive to several 'emotional' stressors in pregnancy, namely restraint and maternal aggression.
- to establish whether attenuated HPA axis responses to emotional stress in pregnancy is a consequence of reduced activation of the CRH and/or AVP neurones in the parvocellular region of the PVN.
- to determine whether pregnant rats demonstrate altered HPA axis and oxytocin secretory responses to the 'physical' stressor, immune challenge and to investigate the mechanisms involved, specifically the role of endogenous opioids.
- to determine whether CRH neurones are non-responsive in pregnancy to other excitatory stimuli, specifically those involved in regulating metabolism and arousal.
- to investigate roles for enhanced *slow* and *rapid* glucocorticoid negative feedback in attenuated HPA axis responses to stress in pregnancy.
- to establish whether the HPA axis is less responsive to stress during late pregnancy in mice, as it is in rats.

CHAPTER 2

General Methods

2.1. Animals

All animals were maintained on a 12 hour light- 12 hour dark photoperiod (lights on at 07:00h for rats and 19:00h for mice) under standard conditions of temperature (ambient temperature of 20-22°C) and humidity in the Medical Faculty animal facility at Edinburgh University, unless otherwise stated. Food and water was available to all animals *ad libitum*. Animals were given at least 1-2 weeks of acclimatisation to animal quarters prior to any experimental procedures or mating. For pregnant groups virgin females were mated overnight with a sexually experienced stud male. The presence of a vaginal plug of semen in the mating cages the following morning was designated day 1 of pregnancy. All procedures performed on animals were approved by the Home Office under the Animals (Scientific Procedures) Act 1986.

2.1.1. Rats

Female Sprague-Dawley rats (Bantin & Kingman) were used throughout these experiments, unless otherwise specified. On arrival at the animal unit rats weighed between 230-260g. Rats were initially housed in groups of 5-6 until surgery, after which they were caged individually.

2.1.2. Mice

For the mouse experiment female BK-white mice (Bantin & Kingman) were used. On arrival to the animal unit mice weighed approximately 30g. From the day of arrival mice were maintained in groups of 6-8 under a reverse 12h light-dark cycle (lights on at 19:00h). Mice were given at least two weeks to acclimatise to this switch in light-dark cycle before mating, and were then maintained with reverse lighting until the end of the experiment. Mice were caged individually from day 14/15 of pregnancy.

2.2. Anaesthesia

For recovery experiments, rats were anaesthetised in a perspex chamber by inhalation of halothane (3% halothane in 600ml/min each of oxygen and nitrous oxide), and anaesthesia maintained throughout by inhalation.

2.3. Surgery

For recovery experiments surgery was performed under sterile conditions using autoclaved instruments; fur was shaved over surgical incision sites.

2.3.1. Cannulation of the jugular vein

The rat was laid on its back and a small skin incision was made between the angle of the mandible and the clavicle (rostro-lateral to the right thoracic nipple). The subcutaneous fat was displaced until the right external jugular vein was located. The connective tissue was gently teased away and once a length of vein had been freed two ligatures (EP1 suture silk) were placed loosely around the vein. The cranial ligature was tightened to prevent venous return from the head. A small 'v-shaped' incision was made in the vein into which a silastic cannula (bore= 0.5mm; wall= 0.25mm: Altec), 12cm in length and filled with heparinised saline (50 units/ml) was inserted. The cannula was guided approximately 3cm into the length of the vein (at which point its tip should meet the atrium of the heart) until blood could be freely withdrawn with a 1ml syringe. The second ligature was tightened to secure the cannula in place and prevent blood loss, then a third ligature was used to secure the cannula tightly to the vein. Once secured, the cannula was exteriorised through a small incision just behind the ears on the back of the neck. The cannula was closed off with a blocked stainless steel connector and the exposed portion was secured with adhesive tape sutured to the skin. Both wounds were closed with sutures (EP3).

2.3.2. Implantation of intracerebroventricular (i.c.v.) cannulae

Rats were implanted with an i.c.v. cannula for drug administration 5-6 days before the experiment. The rat was positioned in a stereotaxic frame and a midline incision was made through the scalp. The scalp and connective tissue were retracted to expose the dorsal surface of the skull and bregma. The i.c.v. guide cannula was aimed at the right lateral ventricle through a hole drilled in the skull (stereotaxic co-ordinates: 1.6mm lateral and 0.6mm posterior to bregma. The guide cannula was a 22-gauge, stainless steel tube (Plastics One) which was inserted to a depth of 4mm from the surface of the skull. The cannula was secured in place with dental acrylic keyed onto the surface of the skull with two jewellers screws. Finally, a dummy cannula (Plastics One) was inserted into the guide cannula and the skin was sutured. After the surgery the rats were handled, dummy caps rinsed in 0.9% saline and loosened and tightened each day to familiarise them with the procedure prior to the day of the experiment and thus reduce non-specific stress responses.

2.4. *In situ* Hybridisation

Since its conception in 1969 (Pardue & Gall, 1969; Gall & Pardue, 1969), *in situ* hybridisation (ISH) has been comprehensively applied to the detection and localisation of specific nucleic acid sequences within a wide range of tissue preparations. This approach offers obvious advantages over other molecular biology techniques since it provides precise information about the site of gene expression at the single-cell level, and can be quantified. The principle behind ISH is the specific annealing of a labelled (a radioisotope or hapten label) nucleic acid probe to complementary sequences of cellular DNA or mRNA in fixed tissue to form a stable hybrid, followed by visualisation of the probe. A critical requirement of this technique is that the nucleic acid is retained *in situ*, since degradation results in a diminished signal. Deoxyribo- and ribo- nucleases (DNase and RNase, respectively) are the most common source of contamination (for DNA and RNA hybridisation, respectively) therefore all equipment and consumables must be autoclaved and

solutions are prepared using sterile double distilled, diethylpyrocarbonate (DEPC) treated water (wherever possible).

Both types of nucleic acid sequences (DNA or RNA) can be employed as probes for hybridisation to localise specific DNA or mRNA. The advantages and disadvantages of using different probes are summarised in table 2.1.

Type of Probe	*Advantages*	*Disadvantages*
Double-stranded DNA probes	(i) Hybridises to both strands of chromosomal DNA. (ii) Specific sequences may be amplified	(i) DNA-DNA hybrids are the least stable. (ii) Requires denaturation of probe prior to use. (iii) Can self-anneal in hybridisation buffer. (iv) Low yield of labelled probe.
Single-stranded cDNA probes	(i) Re-annealing of probe cannot occur. (ii) Specific sequences may be amplified.	(i) DNA-DNA hybrids are the least stable. (ii) Probe must be purified from template mixture.
Synthetic oligonucleotide probes (~20-50 bases)	(i) Higher specific activity than cloned DNA probes. (ii) Good penetration properties. (iii) Available commercially and relatively inexpensive. (iv) Specific sequences can be used to identify different members of the same gene family. (v) "Oligo-cocktail" can be used to amplify signal.	(i) Incorporation of label per molecule of probe is low. (ii) The shorter the probe the lower the stability of the hybrids formed.
Single-stranded RNA probes	(i) High specific activity. (ii) High stability of RNA-RNA hybrids. (iii) Negative control (sense) probe also produced.	(i) Strict RNase free working conditions vital.

TABLE 2.1. Advantages and disadvantages of DNA and RNA probes.
Adapted from Beesley, 2001.

2.4.1. Materials

(i) Microscope Slides

Slides (super premium twin-frost; BDH, Merck House, Poole, Dorset BH15 1TD, UK) were washed overnight in chromic acid and the next day rinsed in cold running water for 2-3 hours. The slides were then passed through a series of rinses: 3 x autoclaved double distilled water (ddH_2O), 1 x DEPC treated water and 1 x 80% alcohol.

(ii) Glassware and Plasticware

All glassware and metal slide racks were wrapped in foil and then baked in an oven overnight at 200°C. Plasticware (eppendorf tubes etc) was autoclaved for 20 minutes at 121°C and 1.7 bar. Items unsuitable for autoclaving were washed thoroughly in dilute Teepol (BDH).

(iii) General Solutions

Solutions were made using DEPC treated double distilled water (ddH_2O) and autoclaved wherever possible. High purity or molecular biology grade reagents were used.

Chrome alum gelatine subbing solution

2.25g gelatine [BDH]
0.23g chromic potassium sulphate [BDH]
1000ml DEPC ddH_2O

Chromic acid

100g potassium dichromate [Sigma]
10ml concentrated sulphuric acid
1000ml ddH_2O

DEPC-treated water

0.1% diethyl pyrocarbonate (v/v) [Sigma]

1000ml ddH_2O

4% Paraformaldehyde in 0.1M PBS; pH 7.2-7.4

4% (w/v) paraformaldehyde [Sigma]

0.1M PBS

0.1M Phosphate buffered saline (PBS); pH7.2-7.4

11.5g di-sodium hydrogen orthophosphate (Na_2HPO_4) [BDH]

2.96g sodium dihydrogen orthophosphate 2-hydrate ($Na_2HPO_4.2H_2O$) [BDH]

8.5g sodium chloride [BDH]

1000ml DEPC ddH_2O

20 x Standard Sodium Citrate (SSC) stock solution

175.4g NaCl [BDH]

88.2g tri-sodium citrate [Sigma]

1000ml sterile ddH_2O

To make 1 x SSC we used 50ml of stock + 950ml ddH_2O.

Triethanolamine (TEA)/acetic anhydride solution

2.98ml triethanolamine [Sigma]

0.50ml acetic anhydride [Sigma]

840µl concentrated hydrochloric acid OR 1.8g NaCl [BDH]

200ml sterile ddH_2O.

(iv) Hybridisation Buffer

Hybridisation buffer was prepared from twelve components, for which details are given below. Directions for preparation of the hybridisation buffer can be found in table 2.2.

Denhardt's Solution

0.5g ficoll [Sigma]

0.5g polyvinylpyrrolidone [Sigma]

1g bovine serum albumin [Sigma]

50ml DEPC ddH_2O

Then passed through a 0.2μm acrodisk filter.

1M Dithiothreitol (DTT)

154 mg dithiothreitol [Sigma]

1ml DEPC ddH_2O

After 30 min DTT was filtered through an acrodisk.

N.B. DTT was not added to the hybridisation buffer until the day of use.

Ethylenediaminetetraacetic acid (EDTA) 250mM

23.26g EDTA [Sigma]

250ml sterile ddH_2O

The pH was adjusted to 8.0 using NaOH.

Formamide (50%)

We used 25ml of 100% stock per 50ml hybridisation buffer. [Sigma]

Poly (A)

15mg poly (A) [Sigma]

1ml DEPC ddH_2O

Salmon testes DNA

50mg salmon testes DNA [Sigma]

5ml sterile ddH_2O

After dissolving the salmon testes DNA it was agitated for 2 hours and then sheared by passing through a sterile 19-gauge needle eight times. The tube was placed into a beaker of boiling water for 5 min and then cooled to room temperature.

5M Sodium Chloride (NaCl)

14.6 g NaCl [BDH]

50ml DEPC ddH_2O.

Sodium pyrophosphate (NaPPI)

0.5g NaPPI [Sigma]

10ml sterile ddH_2O.

Tris pH 7.6

2.98g Trizma base [Sigma]

20ml sterile ddH_2O

The pH was adjusted to 7.6.

Yeast tRNA

100mg yeast tRNA [Sigma]

4ml DEPC ddH_2O

Yeast total RNA

60mg yeast total RNA [Sigma]

3ml DEPC ddH_2O

A 250µl aliquot was boiled for 10 min before adding it to the hybridisation buffer.

Reagents	*Concentration of stock*	*Volume of stock added to hybridisation buffer*
Denhardt's Solution		1ml
Dextran Sulphate	500mg/ml	5ml
Diothiothreitol*	154mg/ml	550µl
EDTA 250mM	93mg/ml	400µl
Formamide	100%	25ml
Poly (A)	15mg/ml	330µl
Salmon testes DNA	10mg/ml	1ml
NaCl 5M	292mg/ml	12ml
5% NaPPI	50mg/ml	500µl
Tris pH7.6	149mg/ml	1ml
Yeast tRNA	25mg/ml	200µl
Yeast total RNA	20mg/ml	250µl

TABLE 2.2. Preparation of 50ml of hybridisation buffer.
The appropriate amounts of each of the components of the hybridisation buffer were added to a sterile tube and made up to 50ml using sterile ddH_2O. *DTT was added immediately prior to use.

(v) Probes

Synthetic oligonucleotide probes purified by a high performance salt free (HPSF) process (MWG-Biotech UK Ltd.) were employed throughout. Oligonucleotides are particularly attractive for use in ISH since they are commercially available and can be produced in large quantities with a high level of purity. Since they are relatively small they are readily accessible to the target and have good penetration properties, a factor which is considered to be important for successful ISH (see table 2.1. for summary).

(vi) Probe specificity

Oligonucleotide probes for ISH were generally selected by consulting the literature to find a previously validated probe sequence for the RNA of interest. Where a probe sequence for the RNA of interest had not already been published the sequence of the gene of interest was found using a web-based search program

(www.ncbi.nlm.nih.gov/Entrez/). A sequence (approximately 36-45 bases long) complementary to a region within the exon (when mRNA was to be detected) or the intron (when hnRNA was to be detected) with a GC content > 60% was selected and the specificity checked (see below).

In both cases the specificity of the probe to be used was checked by performing a web-based "BLAST search" (www.ncbi.nlm.nih.gov/BLAST). BLAST® (Basic Local Alignment Search Tool) is a set of similarity search programs designed to explore all of the available sequence databases (including the rat, mouse and human genome) regardless of whether the query is protein or DNA. The BLAST search results show all the sequences in which there is an overlap with the sequence of interest and provide information about the species, which gene(s) the sequence corresponds to and the percentage match, details of the exact location of the sequence on the gene and whether the sequence is complementary to (plus/minus) or identical (plus/plus) to the gene.

2.4.2. Methodology

To prevent any contamination gloves were worn throughout the ISH procedure.

(i) Tissue Collection and Sectioning

At the end of each experiment animals were killed by decapitation. The brains were rapidly removed, placed on aluminium foil on dry ice and covered with powdered dry ice. Once completely frozen the brains were stored in pre-labelled plastic bags at -70°C. Coronal brain sections (15μm) containing regions of interest (usually PVN) were cut using a cryostat at -16°C (Bright Instrument Co. Ltd). Whilst cutting, the location of all areas of interest was confirmed with sections (1 in 6) stained with toluidine blue in consultation with a stereotaxic rat brain atlas (Konig & Klippel 1963; Paxinos & Watson, 1996). Sections were thaw-mounted (2-3 sections/slide) directly from the knife onto chrome alum subbed microscope slides and stored at -70°C until use for specific ISH analysis.

(ii) 3' end labelling of oligonucleotide probes

In each experiment probes were labelled using a radioactive isotope. [^{35}S] (as [^{35}S]-dATP) was used since this has been found to give good resolution within a fairly short exposure time.

Terminal deoxynucleotidyl transferase (TdT; an enzyme which catalyses a template independent addition of deoxyribonucleoside triphosphates to the 3'-OH ends of double or single stranded DNA) was used to label the 3' end of the probe with radiolabelled nucleotides (poly-A tail).

Labelling Reaction:

27µl	sterile ddH_2O	
10µl	5x TdT Tailing Buffer	[Boeringer-Mannheim]
5µl	25mM Cobalt chloride	[Boeringer-Mannheim]
5µl	^{35}S dATP	[Amersham/NEN]
2µl	Probe (10pmol/µl)	[MWG-Biotech]
1µl	TdT enzyme (25 units/µl)	[Boeringer-Mannheim]

The reaction was incubated in a sterile eppendorf tube at 37°C for 1 hour. Cooling the eppendorf tube on ice stopped the reaction.

(iii) Purification of labelled probe.

QIAquick (Qiagen) spin columns were used to separate labelled probe from the free label and remove DNA fragments (< 10 bases), enzymes, salts and incorporated nucleotides. The reaction mixture (50µl) was added to a spin column together with 500µl of PN (binding buffer) buffer (from kit), the column was centrifuged for 1 min at 6,000 rpm and the radioactive flow-through discarded. Next, 500µl of PE (ethanol wash buffer) buffer (from kit) was added to the column, which was then centrifuged for an additional minute at 13,000 rpm. To elute the labelled probe 50µl of elution buffer (10mM TrisCl; pH 8.5) was added to the centre of the spin column so that it

completely covered the resin filter and allowed to stand for 1 minute. The column was then centrifuged for 1 minute at 13,000 rpm and the eluted solution was collected into a sterile Eppendorf tube. A 1µl sample of the eluted solution (containing the labelled probe) was added to a scintillation vial containing 3.5 ml Ultima Gold scintillation fluid (Packard Bioscience) and then counted for 1 minute in a β-scintillation counter. Incorporation of ^{35}S-dATP was acceptable if the radioactivity was greater than 300,000cpm. The labelled probe was stored at -20°C until hybridisation.

(iv) Tissue Fixation and Prehybridisation

The sectioned tissue has to be pre-treated to optimise the efficiency of hybridisation (McCabe & Pfaff, 1989). Slides for ISH were selected with the aid of marker slides and then transferred to a separate box (inside the freezer) and sealed with tape. On the day of hybridisation the slides were removed from the -70°C freezer and allowed to warm for ~2 hours until they reached room temperature.

Slides were placed in racks and fixed in 4% (w/v) paraformaldehyde in 0.1M PBS (pH 7.2-7.4) for 10 mins, (paraformaldehyde is a cross-linking fixative and generally preferred for optimum retention of cellular RNA, (McCabe & Pfaff, 1989)). Next, the sections were washed twice in 0.1M PBS (5 mins each time), followed by acetylation in triethanolamine/acetic anhydride solution for 10 mins to increase tissue permeability and reduce non-specific probe binding to positively charged amino groups. The sections were then dehydrated through 70%, 95% and 100% ethanol (3min each), delipidated in chloroform for 3 mins, dehydrated in 100% ethanol and partially rehydrated in 95% ethanol (3mins). Slides were then air dried thoroughly.

(v) Hybridisation

The hybridisation procedure performed was adapted from a previously described protocol (da Costa *et al*, 1997). After air drying the slides were arranged on glass platforms in a humidified chamber containing filter paper soaked in ddH_2O. The

labelled probe was diluted in hybridisation buffer, so that a 45µl aliquot of buffer diluted probe would contain 200,000 cpm of activity (if a slide had more than two brain sections the dilution was adjusted so that the hybridisation buffer contained 100,000 cpm/section). Dithiothreitol (DTT) was added (1µl per 90µl of hybridisation buffer) to reduce background, by protecting the sulphur from oxidation. 45µl of hybridisation buffer (containing radiolabelled probe) was pipetted onto each pair of sections/slide. A parafilm "coverslip" was positioned over the sections to ensure the mixture was evenly distributed across the tissue. Chambers were then sealed and incubated at 37°C overnight (approximately 16-18 hours).

In each ISH run positive and negative control slides were included. Positive controls were used to indicate the success of the ISH and were brain sections (containing the region of interest) from rats treated with 1 ml 1.5M NaCl i.p. and killed by decapitation at the appropriate time after the injection for the transcript of interest (i.e. 4h for CRH mRNA and 30 min for NGFI-B mRNA). To confirm that hybridisation of the labelled probe was specific, negative control sections were either hybridised in the same way as above with sense oligonucleotide probes or unlabelled antisense probes (no ^{35}S was included in the labelling reaction).

(vi) Post Hybridisation

After the hybridisation process, slides were individually rinsed in 1 x SSC to remove the Nescofilm "coverslips". Slides were then briefly washed three times in 1 x SSC at room temperature to remove excess buffer and unhybridised probe. Next the slides were placed in metal slide racks and washed for 4 x 15min washes in 1 x SSC at 50-70°C (the temperature of these washes is usually 20-25°C below that of the melting temperature of the probe) followed by a further 2 x 30 min wash at room temperature. Slides were rinsed in ddH_2O and air-dried.

(vii) Hybrid detection

The final step of ISH is the detection of the labelled probe in the tissue. Once dry the slides were placed in autoradiographic cassettes and exposed to Hyperfilm-β-max autoradiography film (Amersham) together with ^{14}C standard microscales (Amersham) for 2-4 weeks (dependent on the probe) at room temperature. Exposed film was developed in D19 developer (Kodak) for 5 mins, rinsed in tap water and then fixed (Ilford Hypam rapid fixer) 2 x 5 mins.

Once the slides had been exposed to film they were dipped in liquid autoradiographic emulsion for the cellular localisation of the RNA of interest. The emulsion (Kodak, type NTB-3) was warmed in a waterbath to 43°C until molten and then under safe light conditions slides were dipped into the emulsion and air dried for 2 hours. Once the slides were dry they were transferred to light tight boxes containing desiccant. The slide boxes were sealed with black electrical tape and wrapped in foil and stored at 4°C for up to 10 weeks (again depending on the probe). Once exposed for the required time slides were developed and fixed in a similar manner to the film. Sections were then counterstained using haematoxylin and eosin, dehydrated in a graded ethanol series, cleared in xylene and coverslipped using DPX mountant.

(viii) Quantitative Analysis

In each case the negative control sections showed no signs of hybridisation, indicating the specificity of the oligo-probe used. The relative amount of RNA in a brain area was assessed using a light microscope (Wang 6000 series microscope) connected to a CCD video camera and the NIH image analysis system (version 1.62) on an Apple MacIntosh computer. For autoradiographic films exposed to the brain tissue, a frame was drawn around the nucleus of interest and grain areas were measured (for pPVN measurements the area was divided as shown in figure 2.1.). Background grain area measurements (6 per section) were made over areas adjacent to the region of interest. The areas used for background analysis were not different

between sections hybridised with the antisense probe and the control sections (i.e. those hybridised with the sense or unlabelled probe). The value of grain area per mm^2 was obtained by subtracting the mean background grain area from each specific nucleus measurement, then dividing the resulting figure by the area of the counting frame. The films were viewed under x 2.5 or x 4 objective depending on the area being measured. For each experiment measurements were taken from at least 3-6 sections (ie. 6-12 PVN profiles).

Emulsion dipped sections were also used for cellular analysis of RNA. This method of analysis involved counting the total number of positive expressing cells in the PVN (of known area). Ten cells adjacent to the nucleus of interest were selected for background measurements and the number of silver grains over these cells counted. A positive cell was defined as one which expressed more silver grains than the mean background + 3 standard deviations. The measurements were then converted to number of positive cells/mm^2.

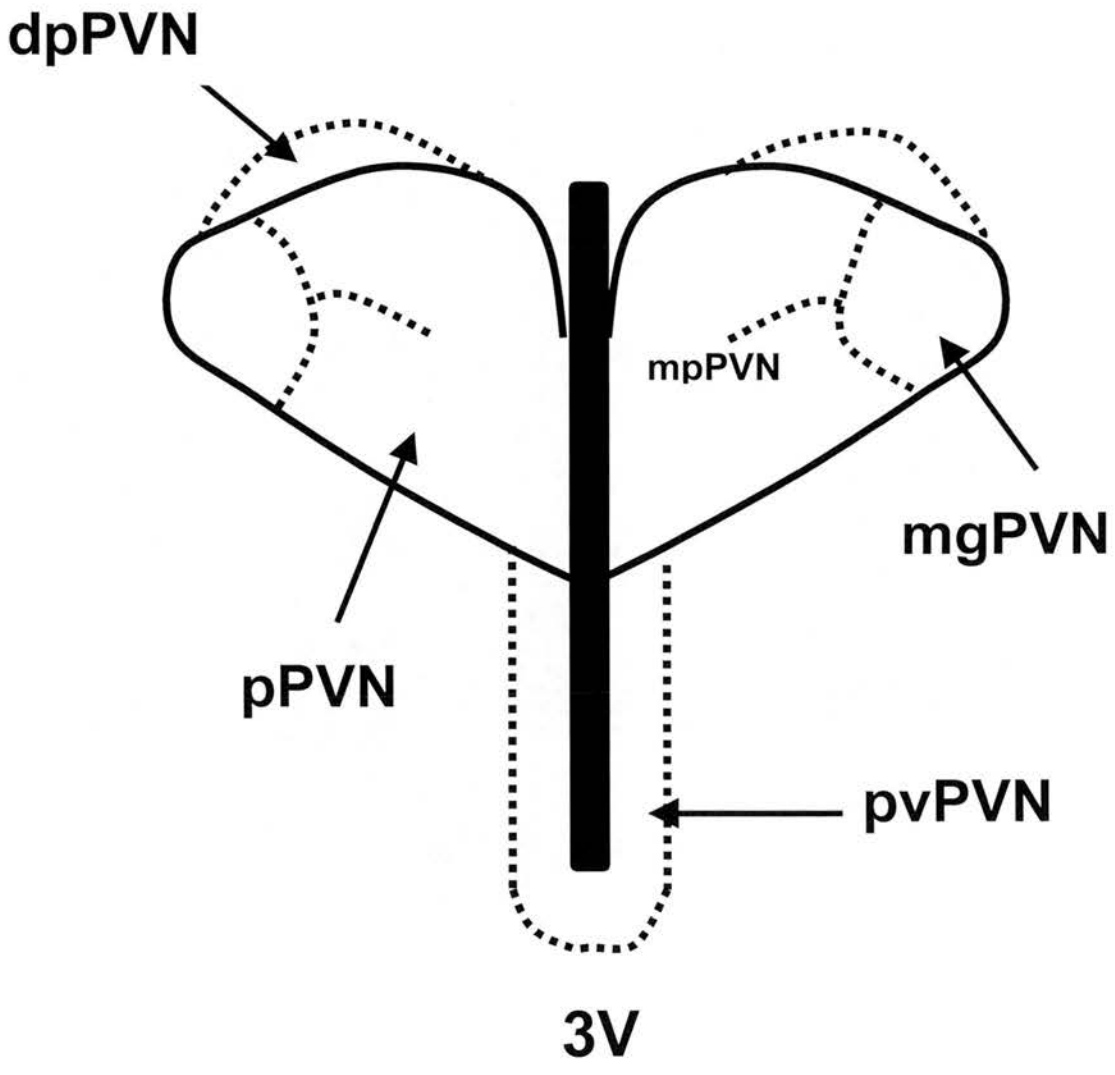


Figure 2.1. Diagram to indicate the subdivisions of the paraventricular nucleus (PVN)

Cells and grain density measurements were made in the parvocellular region of the PVN (pPVN). 3V, third ventricle; pvPVN, periventricular PVN; mgPVN, magnocelluar division of PVN; dpPVN, dorsal parvocellular PVN; mpPVN, medial parvocellular PVN.

2.5. Immunocytochemistry

Immunocytochemistry is a widely used technique first described by Coons *et al* (Coons *et al*, 1941) which enables the detection of specific antigens in a biological sample. It exploits the specific binding of an antibody to its antigen. The reaction is localised in the tissue by attaching a microscopic marker such as an enzyme (e.g. peroxidase) or alkaline phosphatase to the antigen-antibody complex. Many macromolecules (e.g. proteins, carbohydrates, nucleic acids and lipids) can act as successful antigens for raising antibodies. Both polyclonal and monoclonal antibodies can be used for immunocytochemistry. Polyclonal sera contain a mixture of high affinity antibodies, each active against different epitopes on the antigen, whereas monoclonal antibodies are specific to a single epitope on the antigen. There are numerous immunocytochemical techniques that may be used to localise antigens. Here the 'two step indirect method' has been employed. This is where an unlabelled primary antibody reacts with the antigen and is visualised by a labelled secondary antibody targeted against the immunoglobulin of the animal in which the primary antibody has been raised (figure 2.2.). The indirect method is more sensitive than the direct method (where a labelled primary antibody reacts directly with the antigen in the tissue preparation), since several secondary antibodies are capable of reacting with distinct antigenic sites on the primary antibody, therefore more labelled secondary antibody binds to the primary antibody thus amplifying the signal (Beesley, 2001).

2.5.1 Fos Immunocytochemistry

Fos is the protein product of the immediate early gene (IEG) *c-fos* and can be detected by immunocytochemistry using either the indirect method (described in(Johnstone *et al*, 2000)) or the avidin-biotin complex (ABC) (described in (Srisawat *et al*, 2000)) method. Here Fos immunoreactivity was localised using a rabbit affinity purified polyclonal antibody: *c-fos* (Ab-2; Oncogene research products). The antibody was raised against the peptide corresponding to residues 4 to

17 of human Fos. The Fos immunoreactivity was visualised using a labelled secondary antibody: goat anti-rabbit IgG peroxidase (Vector Laboratories). Diaminobenzidine (DAB) was used as the substrate for the enzyme reaction, which converts a colourless chromagen into coloured end products, enhanced by nickel-glucose.

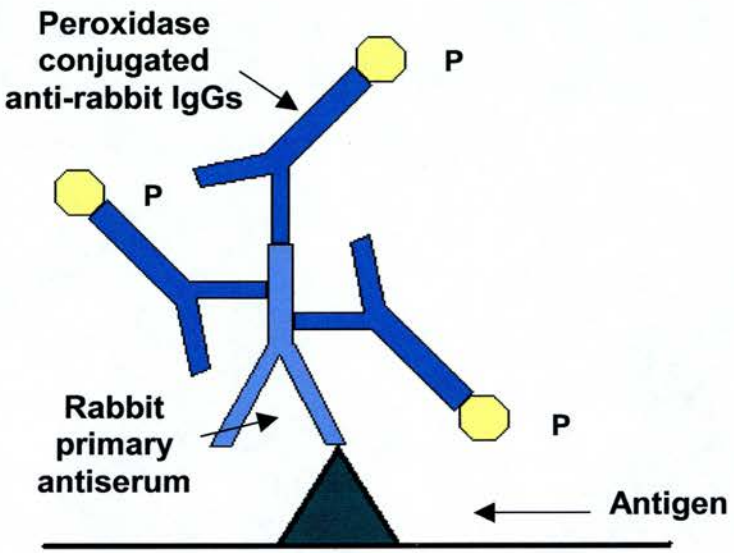


Figure 2.2. Two step indirect immunolabelling method.
Adapted from (Beesley, 2001).

2.5.1.1. Materials

(i) Microscope Slides

Microscope slides were purchased from BDH and coated using a chrome alum gelatine solution (as before except using distilled water instead of DEPC water).

(ii) General Solutions

All solutions were made using single distilled water (dH_2O).

4% Paraformaldehyde

4g Paraformaldehyde [Sigma]

1.15g disodium hydrogen orthophosphate (Na_2HPO_4) [BDH]

0.296g Sodium dihydrogen orthophosphate-2-hydrate ($NaH_2PO_4.2H_2O$) [BDH]

0.85g Sodium Chloride (NaCl) [BDH]

100ml dH_2O

pH to 7.2-7.4

1M Phosphate Buffer (PB)

115g disodium hydrogen orthophophate ($Na_2HPO_4.2H_2O$) [BDH]

27.2g sodium dihydrogen orthophosphate ($NaH_2PO_4.H_2O$) [BDH]

1000ml dH_2O

On the day of use this was diluted 1 in 10 with dH_2O to give 0.1M PB and adjusted to pH 7.2-7.4.

0.1M Phosphate Buffer-Triton X (PB-T)

0.3% (v/v) Triton [BDH]

0.1M PB

Hydrogen Peroxide Solution (to inactivate endogenous peroxidase)

20% (v/v) Methanol [BDH]

0.2% (v/v) Triton-X [BDH]

1.5% (v/v) Hydrogen peroxide [BDH]

50% (v/v) 0.2M PB

Make up with dH_2O.

Preincubation Buffer

1% (v/v) normal sheep serum [Sapu]

PB-T

Primary Antibody Buffer

0.3% (v/v) Triton-X [BDH]
1% (v/v) normal sheep serum [Sapu]
0.02% (w/v) sodium azide [Sigma]
0.1M PB

Primary Antibody Solution

The primary antibody; rabbit polyclonal anti-Fos (*c-fos* Ab-2) [Ongogene] was diluted 1µl/ml in preincubation buffer.

Secondary Antibody Solution

The secondary antibody; goat anti-rabbit IgG-peroxidase [Vector Laboratories] was diluted 2µl/ml in preincubation buffer.

0.2M Acetate Buffer

27.2g Sodium Acetate (CH_3COONa) [BDH]
1000ml dH_2O
Adjust pH 6.0

Nickel-DAB Solution

5g Nickel ammonium sulphate [BDH]
400mg Glucose [Sigma]
80mg Ammonium chloride [Sigma]
50mg Diaminobenzidine (DAB) [Sigma]
3mg Glucose oxidase [Sigma]
100ml Acetate buffer
100ml dH_2O

To make 200ml of the nickel-DAB solution, the nickel ammonium sulphate was first dissolved in 100ml acetate buffer. To this the glucose was added, followed by the ammonium chloride. 1ml of DAB (50mg/ml) was added to 100ml of dH_2O and then

filtered (in the dark, since it is photosensitive). This was then added to the nickel solution and mixed well. The glucose oxidase was added immediately before use.

Stop Solution

The reaction was stopped in a 50:50 solution of 0.2M acetate buffer and dH_2O.

2.5.1.2 Method

Tissue collection and preparation was performed as previously described in section 2.4.2. In brief, rats were killed by decapitation and brains/brainstems were rapidly removed and frozen on dry ice. Brains/brainstems were cut into 15μm coronal sections through the region of interest using a cryostat. Sections were thaw-mounted onto gelatine coated slides and stored at -70°C until immunocytochemical processing.

As with ISH, positive and negative control sections were included for immunocytochemistry (ICC). Positive controls were used to indicate the success of the ICC and were brain sections (containing the region of interest) from rats treated with 1 ml 1.5M NaCl i.p. and killed by decapitation at the appropriate time after the injection (i.e. 90 min for Fos protein). To confirm the specificity of the labelling, negative control sections were included. These were treated in the same way as the experimental sections however the primary antibody step was omitted from the procedure.

Day 1

Slides were removed from the freezer and allowed to reach room temperature. They were then transferred to glass staining dishes and fixed in 4% paraformaldehyde for 30 min at room temperature, followed by 3 x 5 min washes in 0.1M PB. The immunocytochemistry process was performed with gentle agitation on a rotational shaker throughout. Endogenous peroxidase was deactivated with the hydrogen peroxide solution for 15 min and then the sections were washed in PB-T. To prevent

non-specific labelling the sections were incubated in preincubation buffer. After 1 h the slides were transferred to flat slide trays containing filter paper soaked in 0.1M PB. A hydrophobic border was drawn around the sections using a PAP pen (Bayer Diagnostics). The sections were then liberally covered (750µl/slide) with the primary antibody solution containing rabbit anti-Fos (Ab-2) diluted 1:1000 (1µl/ml) in primary antibody buffer. The slide tray were then covered and incubated at 4°C for 48 h.

Day 2

Additional primary antibody solution was applied if necessary to prevent the sections from drying out, and then the slide trays were returned to the refrigerator (4°C) until day 3.

Day 3

The primary antibody solution was drained from the slides, which were then transferred back to the glass staining dishes. The sections were then washed extensively in PB-T (3 x 15min) and returned to the slide trays. The sections were then incubated in an excess (750µl/slide) of secondary antibody solution that contained the goat anti-rabbit peroxidase complex diluted 1:500 (2µl/ml) in preincubation buffer. The trays were covered and stored at 4°C for 24 h.

Day 4

Excess secondary antibody solution was drained from the slides, which were then transferred to the glass staining dishes. The sections were washed 3 x 15 min in 0.1M PB, followed by a 10 min rinse in acetate buffer. Sections were then incubated in the nickel-DAB solution for approximately 10 min. The reaction was monitored by viewing sections under a light microscope and once the Fos nuclei were visualised the reaction was terminated with a 5 min wash in stop solution. The sections were

rinsed in dH_2O, dehydrated in an ascending alcohol series (70%, 90%, 95%, 100%, 100%), cleared in xylene and finally coverslipped using DPX mountant.

Fos positive cells were viewed using a light microscope and manually counted in the region of interest. The area of the region of interest was measured using the NIH image computer program. Data is presented as number of Fos positive cells /mm^2. In each experiment no Fos immunoreactivity was detected in the negative control sections.

2.6. Treatment of Blood Samples

Blood was collected into sterile 1ml syringes containing either EDTA or heparinised saline, depending on intended hormone assay. Blood samples collected for eventual oxytocin radioimmunoassay were collected in syringes containing 80µl heparinised saline (50units/ml) per 1ml of blood. Blood samples collected for eventual ACTH or corticosterone radioimmunoassay were collected into syringes containing 50µl EDTA (5%) per 1ml of blood. All blood samples were stored in eppendorf tubes on ice until centrifugation. Blood was centrifuged at 13,000 rpm for 2-3 minutes. Plasma was separated and stored at -20°C until radioimmunoasssay. In each experiment, blood withdrawn during sampling was replaced with an equal volume of sterile 0.9% saline.

2.6. Radioimmunoassays

Quantitative determination of plasma hormone concentrations was made by radioimmunoassay for oxytocin and corticosterone and an immunoradiometric assay for ACTH (see section 2.7.). The essential principle of radioimmunoassay is based on the ability of a limited concentration of primary antibody to bind a fixed amount of radiolabelled ligand. Increasing amounts of unlabelled antigen in the test sample to be assayed progressively inhibit the binding of labelled antigen to the primary antibody, since they must compete for specific binding sites on the antibody. Therefore, the percentage of bound radiolabelled antigen decreases as a function of the increasing concentration of unlabelled antigen in the sample, thus the amount of bound labelled ligand is inversely proportional to the amount of hormone in the sample. Separation of the bound and free radiolabelled antigen is necessary in order to determine the quantity of unlabelled antigen. This is accomplished by the addition of a second antibody directed against the primary antibody. The concentration of hormone in the unknown sample is then determined by comparing the radioactivity of the precipitate (after centrifugation and aspiration) with the standard curve created by measuring radioactivity in samples of known hormone concentration.

(i) Intra-assay coefficient of variation

To assess variation between samples within an assay, stock solutions of known hormone concentration were prepared and measured in duplicate at the beginning and the end of the assay. Intra-assay coefficients of variation are expressed as percentages and were calculated using the following equation:

$$\textbf{Intra-assay variation} = \left[\frac{\textbf{S.D.}}{\textbf{X}}\right] \textbf{ x 100}$$

Where 'X' is the mean of the hormone concentration from all intra-assay tubes of a specific standard and 'S.D.' is the standard deviation around that mean.

ii) Inter-assay coefficient of variation

To assess variation between assays containing samples from comparable experiments, stock solutions prepared to calculate intra-assay variation were frozen at –20°C and incorporated in the next assay. The inter-assay coefficient of variation was calculated using the following equation:

$$\textbf{Inter-assay variation} = \left[\frac{\textbf{S.D.}}{\textbf{X}}\right] \textbf{ x 100}$$

Where 'X' is the mean hormone concentration from inter-assay tubes of a specific standard from all assays and 'S.D.' is the standard deviation around that mean (standards were chosen that best represented the range as samples in the assay).

(iii) Assay Sensitivity

In order to calculate the sensitivity of an assay it is first necessary to calculate the amount of radioactivity bound (B) by each standard as a percentage of the maximum

binding in the assay (Bmax) or %B/Bmax. This is calculated using the following equation:

$$\%B/Bmax = \left[\frac{\text{mean cpm of standard} - \text{mean cpm of NSB}}{\text{mean cpm of Bmax-mean of NSB}} \right] \times 100$$

Where NSB is the average number of counts for the non-specific binding tubes.

A graph is then created of %B/Bmax against the log concentration of the standards (for example see figure 2.3). Assay sensitivity, expressed as % of total binding, is then calculated using the following equation:

$$\text{Sensitivity} = \frac{[Bmax - (2 \times S.D.\ Bmax)] - NSB}{Bmax - NSB}$$

The graph can then be used to convert the sensitivity of the assay from percentage of total binding to a hormone concentration.

2.6.1. Corticosterone Radioimmunoassay

Plasma corticosterone concentrations were quantified using a commercially available radioimmunoassay kit (IDS Ltd.). The corticosterone assay utilises ^{125}I-labelled corticosterone and a specific anti-corticosterone antiserum to determine the levels of hormone in a plasma sample by the double antibody technique. The cross-reactivity of the assay was evaluated by measuring samples containing various other hormones and steroids. The manufacturers evaluated the cross-reactivity of the assay and found it to specifically measure only corticosterone.

2.6.1.1. Materials

A Gamma-B ^{125}I-Corticosterone RIA Kit containing:

(i) Assay Buffer: phosphate buffered saline

(ii) Corticosterone antiserum: rabbit anti-corticosterone

(iii) [^{125}I]-corticosterone

(iv) Second antibody: goat anti-rabbit antiserum

(v) Corticosterone standard: 1000ng/ml corticosterone

2.6.1.2. Method

(1) Eleven serial dilutions of the corticosterone standard were made to give a range of concentrations from 0.5-1000 ng/ml. Three other standards were also prepared: total counts (TC), non-specific binding (NSB) and maximum binding (Bmax).

TC: contained an aliquot of ^{125}I-corticosterone only, to determine the total count of the radioactivity added.

NSB: contained ^{125}I-corticosterone and second antibody but no first antibody or unlabelled corticosterone, to determine non-specific binding.

Bmax: contained ^{125}I-corticosterone, first and second antibody but no unlabelled corticosterone, to determine the maximum % binding of radioactivity.

(2) 100µl of each standard and sample (containing an unknown concentration of corticosterone) was added to duplicate tubes (except TC).

(3) 100µl of [^{125}I] corticosterone was added to all tubes.

(4) 100µl of corticosterone antiserum was added to all tubes except TC and NSB. The tubes were vortex mixed and incubated for 1 hour at room temperature.

(5) 1ml of saline was added to all tubes except TC, and then the tubes were centrifuged at 1500-1600g for 15 minutes at 2-8°C.

(6) The supernatant of each tube (except TC) was aspirated.

(7) The radioactivity of the pellet in each tube was counted for 1 minute in a gamma counter (LKB Wallac 1272 Clinigammma).

The Wallac Ultroterm 2 software automatically generates a standard curve by plotting the average radioactivity (cpm) for each tube against the known concentration of the corticosterone standards and calculates the unknown values in the plasma samples. An example standard curve from the corticosterone assay is shown in figure 2.3.

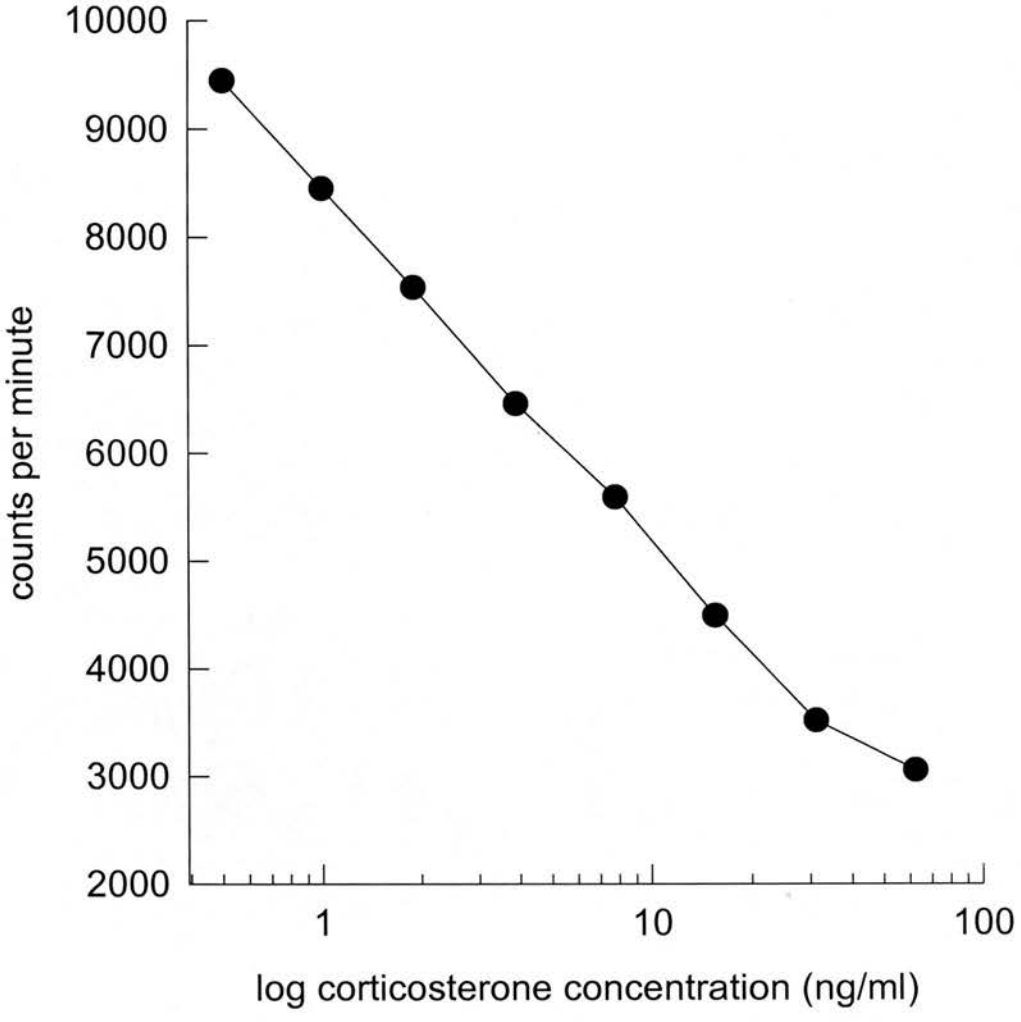


Figure 2.3. An example standard curve from a corticosterone radioimmunoassay. Counts per minute (cpm) are plotted against the known corticosterone concentrations of the standards.

In order to calculate the sensitivity of the assay it is first necessary to construct a graph of %B/Bmax against concentration of the corticosterone standards (as previously described, 2.6.(iii)). An example is given in figure 2.4.

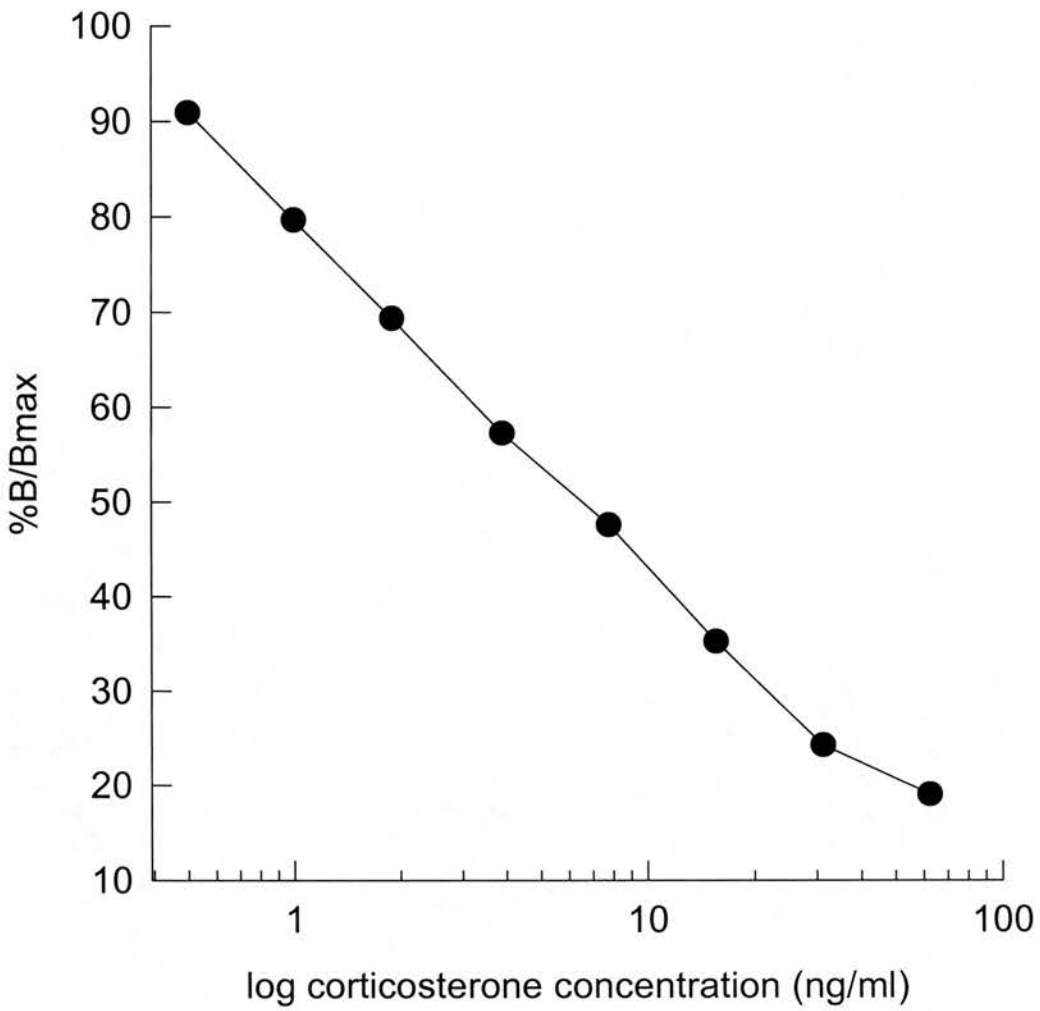


Figure 2.4. Example of binding curve from a corticosterone radioimmunoassay. Percentage of total binding of the standards is plotted against the concentration of corticosterone in each of the standards.

Using the equation detailed in section 2.6. the sensitivity of this assay was calculated to be 83%. From the graph above (figure 2.4.) this is converted into a corticosterone concentration. Thus the sensitivity of the example assay was 0.85ng/ml.

2.6.2. Oxytocin Radioimmunoassay

Plasma oxytocin concentrations were determined using an in-house oxytocin radioimmunoassay. The method employed is adapted from that of Higuchi *et al* (Higuchi *et al*, 1985). They injected a synthetic oxytocin-BSA conjugate into rabbits and raised and purified antibodies against oxytocin. The specificity of the anti-oxytocin serum was determined by comparing the inhibition of binding of ^{125}I labelled oxytocin with that of various other plasma hormones, namely, arginine vasopressin (AVP), arginine vasotocin (AVT), melanocyte-stimulating hormone release inhibiting factor (MIF), luteinising hormone (LHRH), thyrotrophin-releasing hormone (TRH), angiotensin II, rat luteinising hormone, rat follicle stimulating hormone and rat prolactin. The antibody was found not to cross-react with any of the peptides examined. When known amounts of unlabelled oxytocin (range 6.3-400 pmol/l) from male and pregnant female rats were added to serum and measured by this assay recovery was found to be between 94-107%.

A limited concentration of rabbit anti-oxytocin antibody was added to duplicate aliquots of plasma samples (containing unknown concentrations of oxytocin) and to tubes containing the standards (known oxytocin concentration). After 24h incubation, a fixed amount of radio-iodinated oxytocin was added to all the tubes. The tubes were mixed and again incubated (this time for 48h) after which time the amount of ^{125}I-oxytocin bound to the rabbit antibody was inversely proportional to the concentration of oxytocin in the sample. In order to measure the radioactivity of the rabbit anti-oxytocin-^{125}I-oxytocin complex it was separated by precipitation using a secondary antibody technique. Donkey anti-rabbit gammaglobulin was added which binds with the primary antibody; the resulting aggregate was precipitated out of solution with pansorbin cells. Radioactivity of the precipitate was measured with a gamma counter and unknown oxytocin concentrations read directly from the standard curve (created by measuring radioactivity in samples of known hormone concentration) automatically produced by the Ultraterm 2 software package.

2.6.2.1. Materials

(i) Assay (phosphate) buffer

The assay buffer used was prepared in ddH_2O and contained the following:

125mg Sodium dihydrogen orthophosphate ($NaH_2PO_4.2H2O$)	[BDH]
595mg di-sodium hydrogen orthophosphate (Na_2HPO_4)	[BDH]
500mg Sodium azide (NaN_3)	[BDH]
2.5g Bovine serum albumin (BSA)	[Sigma]

(ii) Standard Oxytocin

Standard oxytocin (4th International Standard) was obtained from the National Institute for Biological Standards and Control as a freeze-dried aliquot containing 21μg of oxytocin. This was resuspended in assay buffer to give a 50ng/ml solution and re-frozen as 1ml aliquots.

(iii) First Antibody Buffer

Normal rabbit serum (Sapu) was used in assay buffer at a 1:400 dilution to aid the binding of the secondary antibody.

(iv) First Antibody

The rabbit anti-OXT antibody THF-3 was kindly donated by Dr T. Higuchi. It was stored diluted 1:200 in assay buffer at -20°C. On the day of use, the first antibody solution was used at a final dilution of 1:200,000 in antibody buffer.

(v) Second antibody

The second antibody was donkey anti-rabbit gammaglobulin (IDS Ltd.) It was stored at 4°C and used at a dilution of 1:25 in assay buffer.

(vi) [^{125}I]-Oxytocin

[^{125}I]-OXT (NEN Life Science Products) arrived in a lyophilised form. It was resuspended in assay buffer, aliquoted into 0.5ml eppendorf tubes and stored at -20°C. We used the [^{125}I]-OXT at a concentration calculated to contain between 7-10,000cpm/50µl. This was verified by counting a 50µl aliquot in the β-scintillation counter for 1 minute.

(vii) Standard Pansorbin Cells

Pansorbin cells were supplied by Novabiochem Ltd. as a 10% (w/v) solution in phosphate buffered saline containing 0.1% (w/v) sodium azide. This solution was used at a 1:25 dilution with assay buffer.

2.5.2 Methods

On day 1 of the assay 50µl of rabbit anti-OXT antibody was added to duplicate aliquots of the experimental rat plasma samples (containing unknown concentrations of OXT) and to triplicate tubes containing standard OXT (of known concentration) in assay buffer. The tubes were vortex mixed, covered in foil and stored at 4°C for 24 hours; after which time we added a 50µl aliquot of ^{125}I-OXT (which contained approximately 8-9000 cpm) to all of the tubes. The mixture was left for 48h or over the weekend after which time the amount of ^{125}I-OXT bound to the rabbit antibody was inversely proportional to the concentration of OXT in the plasma sample/standard. Before the radioactivity of the rabbit anti-OXT-^{125}I-OXT complex could be measured it had to be precipitated out of solution. This was achieved by adding 50µl of the secondary antibody; donkey anti-rabbit. The tubes were incubated overnight at 4°C and the folowing morning the precipitate was separated by centrifugation (3000rpm, at 4°C for 30 mins) with white pansorbin cells, which aid visualisation of the precipitate. The resulting supernatant was aspirated and the

radioactivity of each precipitate was then measured for 1 min using a gamma counter (Wallac).

To quantify the unknown OXT concentrations in the plasma samples it was necessary to construct a standard curve of precipitate radioactivity versus standard OXT concentrations (Ultroterm 2 software). On day one of the assay serial dilutions of stock OXT (50ng/ml) were made which created eleven standard concentrations ranging from 2.4-2500pg/ml. In order to determine the sensitivity and binding of the assay three other standards were prepared as below:

Total Count (TC): ^{125}I-OXT only
Non-specific binding (NSB): ^{125}I-OXT + 2nd Antibody
Maximum % binding (B_{max}): ^{125}I-OXT + 1st antibody + 2nd antibody

To measure intra-assay variation duplicate tubes containing either 10, 50, 100, 250 or 500 pg/ml OXT were incorporated at the beginning and at the end of the assay. The remainder was stored at -20°C until the next assay in order to assess inter-assay variation.

An example standard curve generated from an oxytocin assay is shown in figure 2.5.

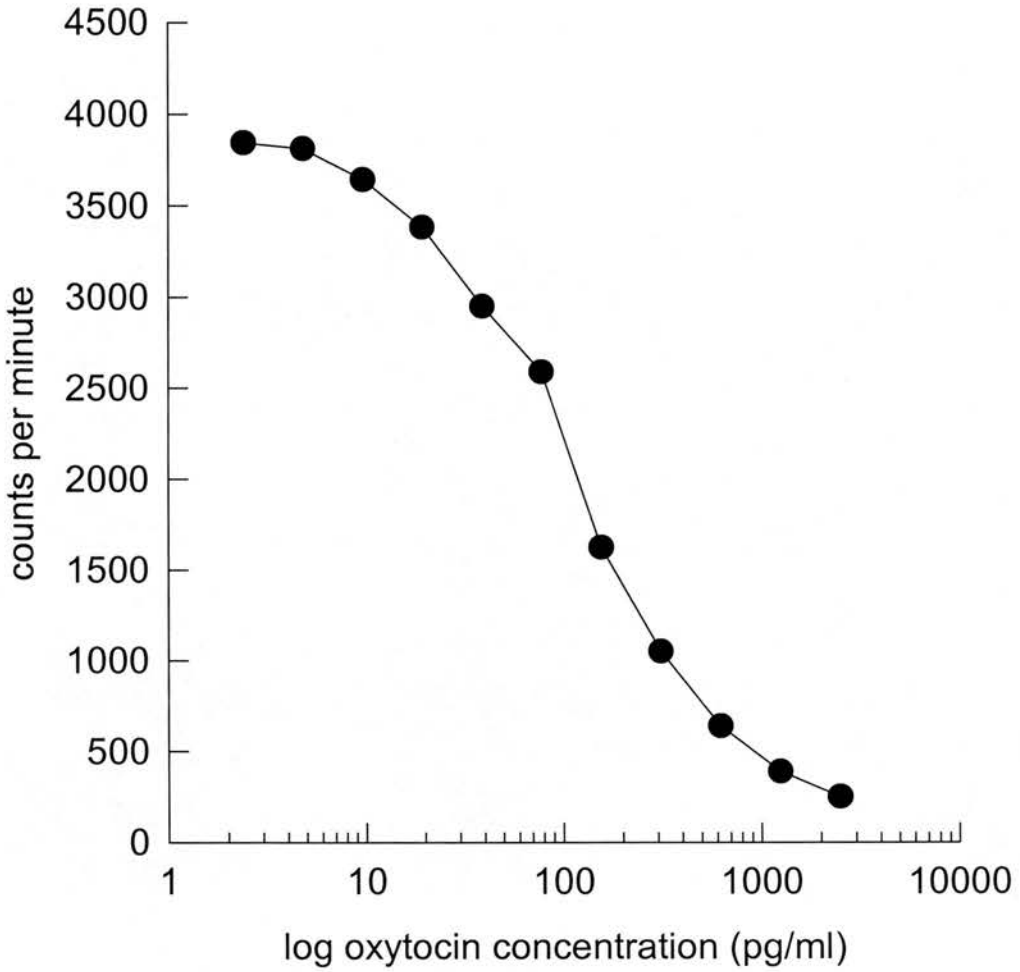


Figure 2.5. Example of a standard curve from an oxytocin radioimmunoassay.

In order to calculate the sensitivity of the assay it is first necessary to construct a graph of %B/Bmax against concentration of the oxytocin standards (as previously described in 2.6.(iii)). An example is given in figure 2.6.

Using the equation detailed in section 2.6. the sensitivity of this assay was calculated to be 96%. From the graph below (figure 2.6.) this is converted into an oxytocin concentration. The sensitivity of the example assay was found to be lower than the first standard of 2.4 pg/ml.

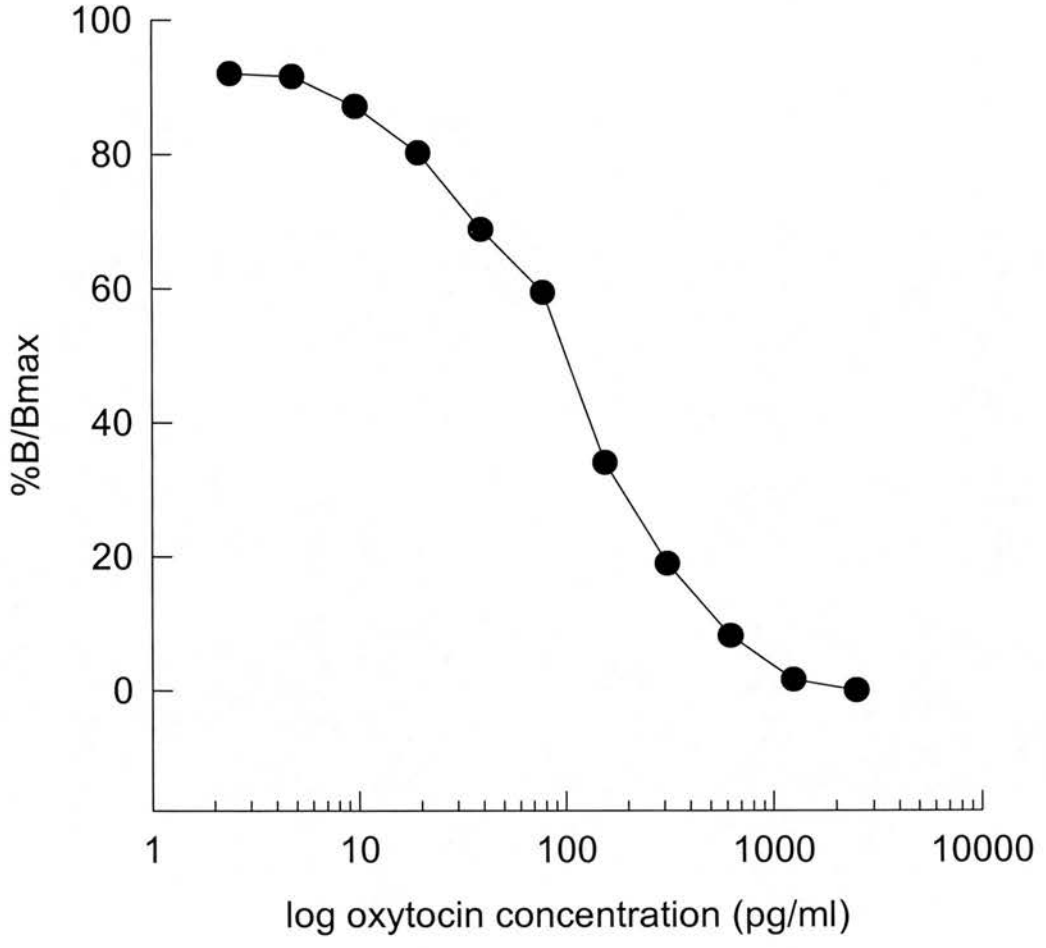


Figure 2.6. Example of binding curve from an oxytocin radioimmunoassay. Percentage of total binding of the standards is plotted against the concentration of oxytocin in each of the standards.

2.7. Adrenocorticotropic hormone immunoradiometric assay

Adrenocorticotropic hormone (ACTH) is a single chain polypeptide consisting of 39 amino acids. The first 24 *N*-terminal amino acids are essential for the hormone's biological activity. Here a commercially available two-site solid phase immunoradiometric (IRMA) kit was employed for the quantitative determination of intact ACTH in plasma (Hodgkinson *et al*, 1984).

Two polyclonal antibodies, recognising different binding sites on the ACTH (rat and mouse) molecule were used in excess. One antibody is a [^{125}I]-labelled sheep immunoglobulin that recognises the amino terminal region of ACTH. The second antibody is immobilised and coupled to the sheep anti-rabbit coated reaction tubes. This antibody reacts with the *C*-terminal of the ACTH molecule. Following overnight incubation a "sandwich-type" complex was formed and bound to the tube wall (see figure 2.7.). The excess [^{125}I]-sheep-anti-ACTH was removed by aspiration. Following two washes the radioactivity in the tubes was measured using a gamma counter (Wallac) and at this time was directly proportional to the concentration of the ACTH in the sample. Recovery was determined by adding known amounts of ACTH to plasma samples and measuring the concentration using this kit. The recovery was found to be between 99-104%. Cross reactivities were determined by the manufacturer by measuring plasma samples containing α-melanocyte stimulating hormone (α-MSH), β-MSH and β-endorphin. The ACTH IRMA was found to specifically measure only intact ACTH.

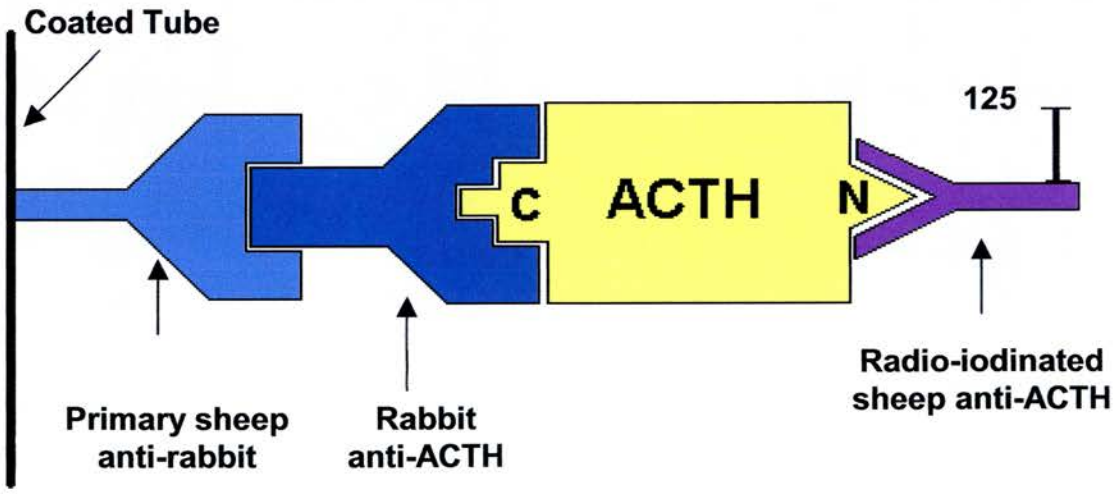


Figure 2.7. Schematic representation of the coated tube ACTH assay.

2.7.1 Materials

An ACTH IRMA kit (IDS Ltd.) containing:

(i) Seven standard ACTH solutions ranging from 0 pg/ml-1250 pg/ml

(ii) Two reference controls containing ~30 pg/ml and ~300 pg/ml ACTH

(iii) [^{125}I]-ACTH antibody (rabbit-anti-ACTH)

(iv) Coated Tubes: C-terminal anti-ACTH coupled to sheep-anti-rabbit coated tubes

(v) Wash Buffer

2.7.2 Method

(1) 200µl of each standard, reference control and plasma sample (containing an unknown concentration of ACTH) were added to duplicate tubes (except total count; TC).

(2) Next, 200µl of [^{125}I] ACTH antibody solution was added to each tube and vortex mixed.

(3) The tubes were incubated overnight at room temperature.

(4) The following day all tubes (except TC) were washed twice with wash buffer.

(5) The excess [^{125}I] ACTH antibody was aspirated.

(6) The radioactivity of each tube was counted for 1 min in a gamma counter (LKB Wallac 1272 Clinigammma).

Wallac Ultroterm 2 software automatically generates a standard curve by plotting the average radioactivity (cpm) for each tube against the known concentration of the ACTH standards and calculates the unknown values in the plasma samples (figure 2.8).

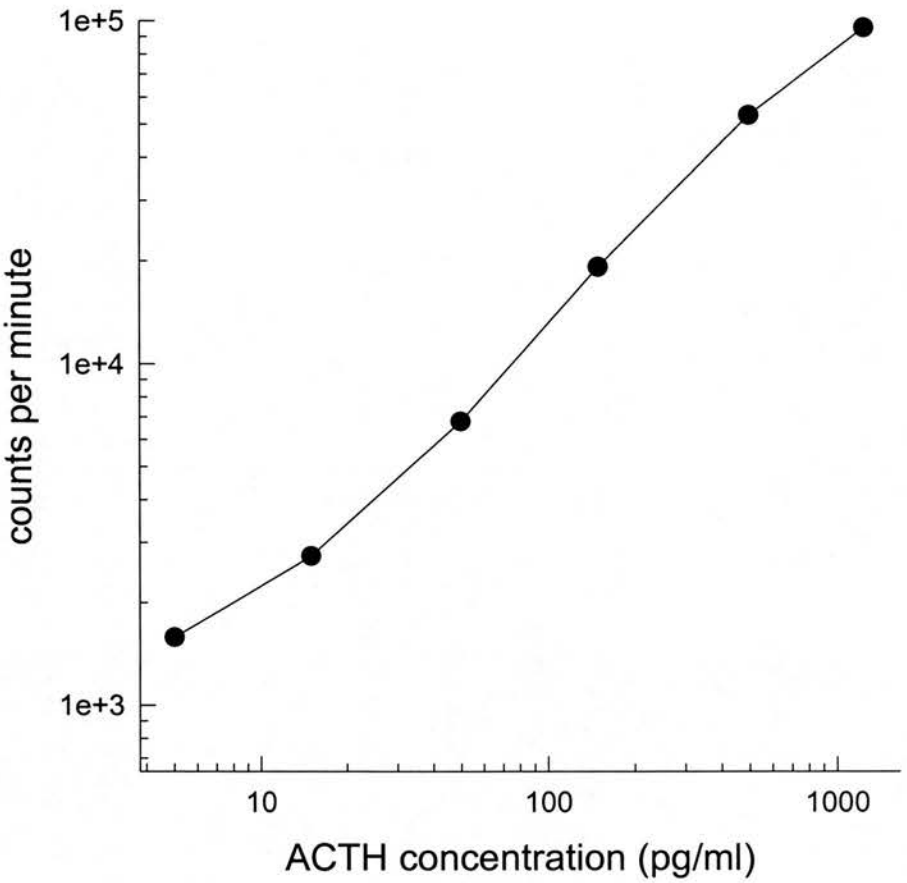


Figure 2.8. Example of ACTH standard curve.

cpm vs ACTH concentration.

The sensitivity of this assay defined as plus 2 standard deviations (i.e. 95% confidence limit) from the zero calibrator is 1.0pg/ml. Intra- and inter- assay variation were assessed as previously described (see section 2.6).

CHAPTER 3

Effects of Emotional Stressors on the Hypothalamic-Pituitary-Adrenal Axis in Pregnancy

3.1. Introduction

It is well established that lactation is associated with attenuated neuroendocrine responses to stress, including reduced ACTH, corticosterone (Windle *et al*, 1997), oxytocin (Higuchi *et al*, 1988) and prolactin (Higuchi *et al*, 1989) secretion. Less is known about the physiological adaptations that occur in response to stress in pregnancy. Recently Neumann and colleagues (Neumann *et al*, 1998) reported that the reduced responsiveness of the HPA axis to stress observed in lactation manifests in late pregnancy. Pituitary ACTH secretion and corticosterone release from the adrenal gland are attenuated in pregnant rats following 5 minutes exposure to an emotional stressor, the elevated plus maze. This seems to involve adaptations at the level of the anterior pituitary, since *in vivo* the pituitary corticotrophs are less sensitive to stimulation by exogenously administered CRH in pregnancy and CRH is less effective in evoking cAMP production (Neumann *et al* , 1998) from isolated pituitary segments from late pregnant rats; a reduction in pituitary CRH binding sites is likely to be involved (Neumann *et al* , 1998).

The changes that occur at the level of the anterior pituitary may account for the attenuated HPA axis responses in pregnancy, however it is likely that changes at the level of the hypothalamus may also contribute to the hyporesponsiveness of the HPA axis to stress at this time. CRH mRNA content in the parvocellular PVN is significantly reduced by day 21 of pregnancy and the CRH mRNA: AVP mRNA ratio is unaltered indicating decreased basal production of both CRH and AVP in pregnancy (Johnstone *et al*, 2000a). It is not known if stress-induced CRH and/or AVP gene transcription are differentially regulated in pregnant rats. It seems plausible that if the pPVN neurones themselves, or the inputs impinging upon them are less responsive to stress in pregnant animals then this may explain the attenuated responsiveness of the HPA axis in pregnancy. Thus the aim of the present set of experiments was to investigate whether the reduced responsiveness of the HPA axis to stress in pregnancy was a consequence of reduced activation of hypothalamic pPVN neurones.

Restraint Stress

In experiments 1 and 2 the stress paradigm used was exposure to 30 minutes restraint in a perspex tube, since this is a well characterised 'emotional' or 'psychological' stressor which acts as a potent activator of the HPA axis (Ma *et al*, 1997). In non-pregnant rats, acute exposure to restraint stress evokes ACTH and corticosterone secretion and increases CRH and AVP mRNA expression in the pPVN (Harbuz *et al*, 1994; Ma *et al* , 1997). Increases in CRH and AVP mRNA expression indicate that stress activates gene transcription in the parvocellular PVN neurones.

Immediate early genes (IEGs) are characterised by their rapid and transient induction in many cell types and their responses are independent of protein synthesis. NGFI-B (nerve growth factor induced gene B) is classified as an immediate early gene and is induced rapidly in neurones by a variety of stimuli, including stress (Chan *et al*, 1993); (Imaki *et al*, 1996) and i.c.v. administration of CRH (da Costa *et al*, 1997). Its rapid transcription means that NGFI-B is a good indicator of neuronal activation. NGFI-B was first identified in the rat phaeochromocytoma cell line PC12, as a gene induced by nerve growth factor (Maruyama *et al*, 1998). NGFI-B belongs to an orphan (so called since specific ligands for these receptors have yet to be identified) subclass of the nuclear steroid receptor superfamily. These receptors are ligand-activated transcription factors which regulate the expression of target genes via binding to specific sequences (known as hormone response elements), in their promoter regions.

C-fos is another IEG that has been extensively studied and like NGFI-B has been identified as a marker of neuronal activation at various levels within the brain, including cells in the parvocellular region of the rat PVN (Wisden *et al*, 1990). Its protein product, Fos, forms a dimer with proteins encoded by *c-jun*. This heterodimer is known to interact with a specific sequence, the AP-1 site to regulate transcription of target genes (Chan *et al* , 1993). Several studies have shown that Fos is induced in the PVN in response to stress, however the CRH gene promoter does not appear to have a functional AP-1 binding site, suggesting that Fos is not directly

involved in stress induced changes in CRH expression. However, a potential NGFI-B response element has been found in the 5' promoter region of the rat CRH gene (Wilson *et al*, 1991) and similar sequences have been found in the promoter regions of the oxytocin (Ivell & Richter, 1984) and arginine vasopressin (Mohr & Richter, 1990) genes. It is yet to be elucidated whether NGFI-B is capable of binding to the potential NGFI-B response elements (NBRE) identified (in any of the genes mentioned above) and initiating transcriptional activation, however NGFI-B could be a link between neuronal depolarisation and activation of the CRH gene.

In experiment 1 to determine whether attenuated HPA axis responses in pregnancy are a consequence of reduced activation of pPVN neurones, *in situ* hybridisation for NGFI-B mRNA was employed to indicate rapid neuronal activation in hypothalamic pPVN neurones following exposure to restraint stress. NGFI-B mRNA expression was also measured in the hippocampus, since this structure is important in regulating the activity of pPVN CRH and AVP neurones (Herman & Cullinan, 1997). In experiment 2, rats were also exposed to restraint, however this time, brains were processed by *in situ* hybridisation for AVP heteronuclear RNA (hnRNA).

Heteronuclear RNA (hnRNA) represents the primary transcripts of RNA polymerase II and includes precursors of mRNAs from which introns are spliced out. Therefore, measurement of hnRNA expression should reflect the rate of gene transcription more directly than the measurement of stable pools of mRNAs (Ma *et al* , 1997). Heteronuclear RNAs are rapidly processed to mature mRNA in the nucleus and have a high turnover rate meaning they are usually detectable in the cytoplasm at approximately 15-30 minutes after transcriptional activation. Since changes in levels of cytoplasmic mRNA occur over a relatively slow time course, changes in total levels of cytoplasmic mRNA may not correlate well with changes in gene transcription. Therefore when looking at rapid changes in gene transcription following physiological challenge, measuring changes in IEG and heteronuclear RNA expression are perhaps more suitable methods to use.

The aims of experiments 1 and 2 was to establish whether the ACTH secretory response to acute restraint stress (30 min) is attenuated in late pregnancy, and if so, to establish whether this is a result of reduced activation of the CRH/AVP pPVN neurones.

Maternal Defence Test

In most studies investigating HPA activity a variety of stressors have been employed, such as forced swimming (Neumann *et al* , 1998), restraint (Harbuz *et al* , 1994; Ma *et al* , 1997), elevated plus maze (Neumann *et al* , 1998) and noise (Windle *et al* , 1997). Although these stressors are relevant models in that they activate the HPA axis, concerns have arisen that these type of stressors may not be of primary significance to a rat during the later stages of pregnancy. In male rats social defeat has been used as an emotional stressor to activate the HPA axis (Haller *et al*, 1995; Wotjak *et al*, 1996). Social defeat is an emotionally challenging situation that consists of placing the experimental animal (intruder) in the home cage of an adult male rat (resident). The resident is housed with a female prior to the experiment, so as to stimulate territorial aggression (Flannelly & Lore, 1977). Female rats tend to be relatively docile animals and are only territorial and aggressive during lactation, therefore the maternal defence test was devised as a relevant emotional stressor for female rats (Neumann *et al*, 2001). The test relies upon female aggression directed against an intruder that approaches the nest. The aggressive behaviour displayed by the lactating mother is related to the protection and defence of her young and is part of the complex behavioural pattern described as maternal behaviour (Rosenblatt *et al*, 1994).

Therefore the aim of experiment 3 was to establish whether pregnant rats demonstrate attenuated HPA axis responses to a physiologically relevant stressor, namely the maternal defence test. *In situ* hybridisation for NGFI-B mRNA was again employed to study rapid changes in gene transcription in pPVN neurones.

3.2. Materials and Methods

3.2.1. Animals

Female Sprague Dawley rats were used in experiments 1 and 3 and were maintained in the animal facility within the Faculty of Medicine, University of Edinburgh. Experiment 2 was performed in the Max Planck Institute for Psychiatry in Munich (in collaboration with Drs. Alexander Wigger, Inga Neumann and Alison Douglas) and female Wistar rats were used (animal procedures were performed in accordance with the guide for the care and use of laboratory animals issued by the government of Bavaria). In all cases rats were maintained under standard conditions described in chapter 2. For the pregnant groups, rats were mated overnight with a sexually experienced male. The presence of a vaginal plug of semen in the breeding cage the following morning was designated day 1 of pregnancy.

3.2.2. Stress Paradigms

All experiments were performed in the morning between 08:30-11:30h.

Experiment 1: Rats were individually caged at least five days before the start of the experiment. Virgin females and rats on day 10 and day 21 of pregnancy were used. Virgin and pregnant rats were either restrained for 30 minutes in Perpsex restraining tubes (diameter= 70mm, the length of the tubes were adjusted to prevent the animal from turning around) or left undisturbed in their home cages and then killed immediately by decapitation (rats were conscious and no anaesthesia was used). This time point, i.e. 30 min after the onset of stress was chosen since it has been shown to correspond with the peak in NGFI-B mRNA, *c-fos* mRNA and CRH hnRNA (Parkes *et al*, 1993; Imaki *et al* , 1996; Mansi *et al*, 1998) expression in the pPVN. Brains were rapidly removed and frozen on pieces of aluminium foil on dry ice. To aid freezing they were covered with powdered dry ice. Once frozen the brains were stored at -70°C. Trunk blood was collected into chilled plastic tubes contained 5% EDTA (150µl per 1ml of blood). Blood samples were kept on ice until they were

centrifuged (3 min at 13000 rpm) and plasma separated. Plasma samples were then stored at -20°C until assayed.

Experiment 2: Female virgin and day 21 pregnant Wistar rats were used. Rats were caged in pairs of the same reproductive state, 5 days prior to the start of the experiment. From each pair one rat was exposed to 30 minutes restraint stress in a plexiglass tube (diameter 80mm). After the period of restraint, rats were returned to their home cage and left undisturbed for a further 60 minutes, after which time they were killed by decapitation (i.e. 90 minutes after the onset of stress). The control animal from each pair was killed by decapitation immediately prior to or immediately after its 'stressed' partner. Brains were rapidly removed, snap-frozen in methylbutane on dry ice, then stored at -70°C.

Experiment 3: Female virgin and day 21 pregnant Sprague Dawley rats were used.
Maternal defence test: 2-3 days before expected parturition (day 22) seven pregnant rats were transferred into individual cages. After parturition these rats were kept with their litters. This group of rats was used in the experiment to provide the stress stimulus and referred to as the 'lactating residents'. The experiments were performed when the resident mothers were between days 4-6 of lactation (the day after parturition was designated day 1 of lactation). On the morning of the experiment the virgin or pregnant (day 21) intruder was taken from its home cage and placed into the cage of the lactating resident. The intruder rats were exposed to the 'maternal defence test' for 30 minutes, during which time the number of attacks launched upon the intruder rat by the lactating resident was recorded. Each lactating resident was used twice, so each was exposed to intruders of both reproductive states. Thus, if on day 1 the lactating resident was exposed to a virgin intruder, then on day two a pregnant intruder was used, and vice versa. After 30 minutes exposure to the 'maternal defence test' the virgin and pregnant intruder rats were immediately killed by decapitation. Virgin and pregnant control rats were undisturbed, then killed by decapitation. Trunk blood was collected into chilled plastic tubes containing 5% EDTA (150µl per 1ml blood), plasma was separated by centrifugation (as above) and stored at -20°C until radioimmunoassay.

3.2.3. *In situ* hybridisation

Brains were sectioned at 15μm and mounted on slides as previously described in the General Methods Chapter. To detect NGFI-B mRNA expression a 45-mer oligonucleotide probe was used [MWG-Biotech], complementary to nucleotides spanning amino acids 1-15 (see below) (Wisden *et al* , 1990).

5'- GGT GGC AGG TGC GGT GGG CGC CGT CTC GGG GCT GGC CAG GTC CAT -3'

To detect AVP hnRNA expression a 39-mer oligonucleotide probe [MWG-Biotech], complementary to the first 39 bases of intron 1 of the rat AVP gene was employed (see below).

5'- ACG GCA CTG TCA GCA GCC CTG AAC GGA CCA CAG TGG TAC -3'

Both probes were labelled with [^{35}S] as previously described in the General Methods Chapter. Hybridisation and post-hybridisation washes were performed as before. The melting temperature of the NGFI-B mRNA oligo-probe is 85°C and for the AVP hnRNA oligo-probe is 78°C, therefore the heated SSC washes were performed at 65°C and 58°C, respectively. Once dry, the sections were exposed to autoradiographic film in autoradiographic cassettes at room temperature. The exposure time was 21 days for NGFI-B mRNA and 25 days for AVP hnRNA. The relative amount of NGFI-B mRNA or AVP hnRNA in the pPVN was assessed by measuring silver grain density from the autoradiographic film using a computer based image analysis system as described in the General Methods Chapter.

3.2.4. Radioimmunoassays

Plasma ACTH and corticosterone concentrations were determined using commercially available kits (see sections 2.6.1. and 2.7.). For both experiments 1 and

3, the sensitivity of the ACTH assay was 1 pg/ml. The intra-assay variation was < 8% for experiment 1 and <4% for experiment 3. The sensitivity of the corticosterone assay was 0.5ng/ml and the intra-assay variation was <3%.

3.2.5. Statistical Analysis

Two way analysis of variance (2-way RM ANOVA), followed by Student-Newman-Keuls multiple comparison test was used to analyse the data. P values less than 0.05 were considered statistically significant.

3.3. Results

Experiment 1

3.3.1. Effects of restraint on plasma ACTH

Basal plasma concentrations of ACTH did not differ between any of the groups. Following exposure to 30 min restraint a significant increase in plasma ACTH concentration was observed in all 3 groups (see figure 3.1). Further analysis revealed that plasma ACTH concentration in the d21 pregnant group (20.2 ± 2.8 pg/ml) was significantly attenuated ($p<0.001$; Two-way ANOVA) when compared to the levels in the virgin and d10 pregnant groups (36.9 ± 3.9 and 40.65 ± 7.1 pg/ml, respectively). No significant difference was found between the virgin and the d10 pregnant animals following restraint.

3.3.2. Effects of restraint on NGFI-B mRNA expression in the pPVN

Levels of NGFI-B mRNA expression in the pPVN of control animals were not significantly different between any of the groups (figure 3.2.). Exposure to 30 min restraint evoked a marked increase in the expression of NGFI-B mRNA in the parvocellular region of the PVN in virgin (3-fold increase in grain area) and d10 pregnant rats (4.9-fold increase in grain area).

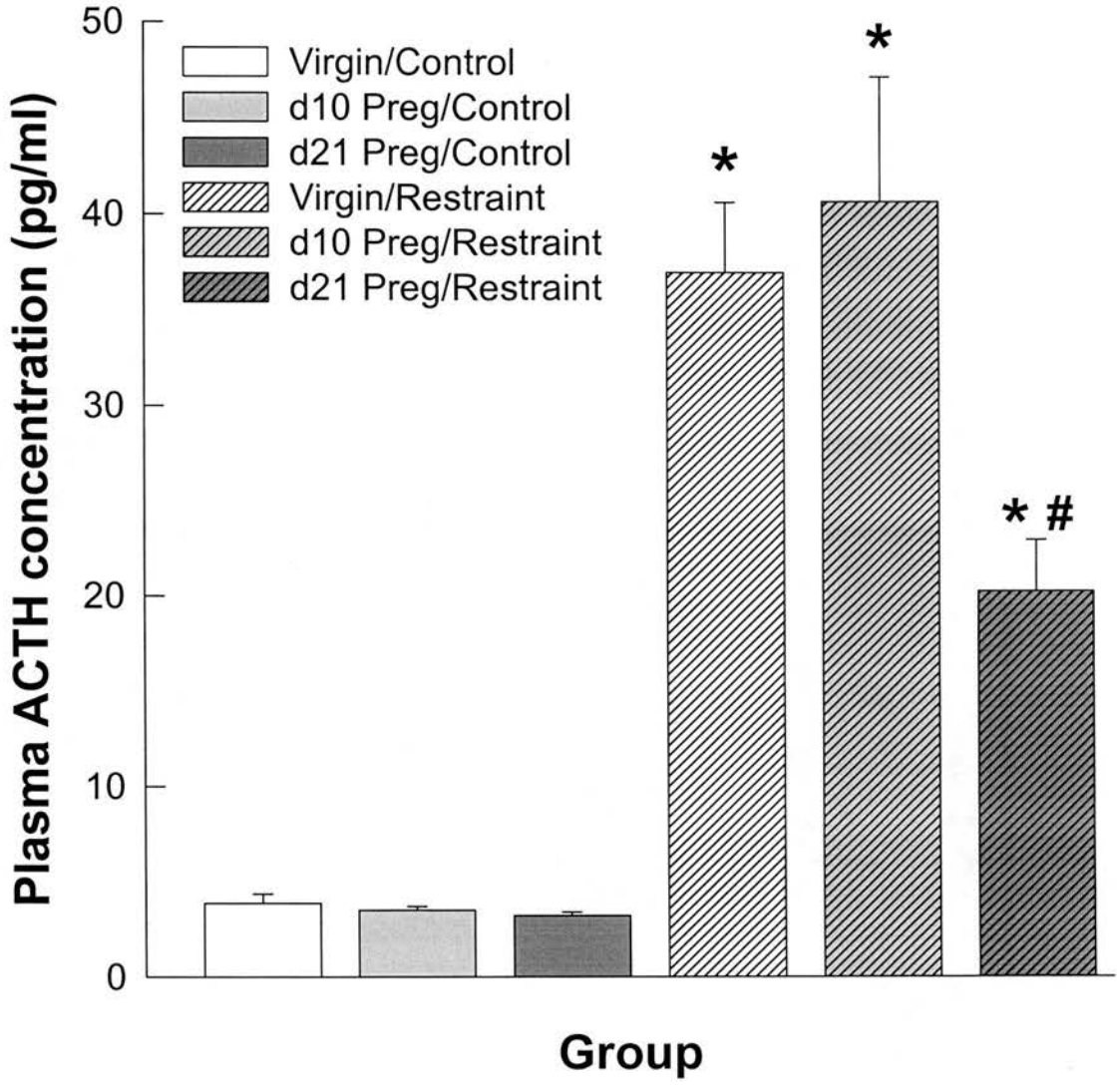


Figure 3.1. The effect of 30 minutes restraint on plasma ACTH concentrations in virgin and pregnant rats.

Trunk blood was collected immediately after the 30 minute period of restraint. Control rats were removed from their home cages and killed immediately. Values are plotted as group means ± SEM. Group numbers: virgin/control, n = 8; day 10 pregnant/control, n = 5; day 21 pregnant/control, n=8; virgin/restraint, n = 10; day 10 pregnant/restraint, n = 6; day 21 pregnant restraint. Two-way ANOVA followed by Student Newman-Keuls multiple comparison test was used for statistical analysis of the data: *$p < 0.001$ vs basal values in the same group; #$p < 0.001$ vs virgin/restraint.

In contrast, only a small non-significant increase was observed in the day 21 pregnant group (1.3-fold increase in grain area; see figure 3.2). A two-way ANOVA revealed a significant effect of restraint stress in both the virgin and d10 pregnant group on NGFI-B mRNA expression, however the increase in expression observed in the d21 pregnant group proved not to be significantly different from basal levels. Statistical analysis across the groups demonstrated that following 30 minutes of restraint, levels of NGFI-B mRNA in the PVN of virgin animals was significantly greater than those seen in animals on day 21 of pregnancy. The levels of expression found for d10 pregnant rats appeared to be intermediate with no significant difference in levels compared with either virgin or d21 pregnant rats.

3.3.3. Effects of restraint on NGFI-B mRNA expression in the hippocampus

Basal expression of NGFI-B mRNA in the hippocampal subfields of control animals were not significantly different between any of the groups (figure 3.3). Following 30 min exposure to restraint, NGFI-B gene transcription showed a tendency to increase only in the virgin group, however this was not significant. Nevertheless, after restraint, the NGFI-B mRNA signal was found to be significantly less in the CA2, CA3 subfields and dentate gyrus in the day 21 pregnant rats compared with the virgin rats.

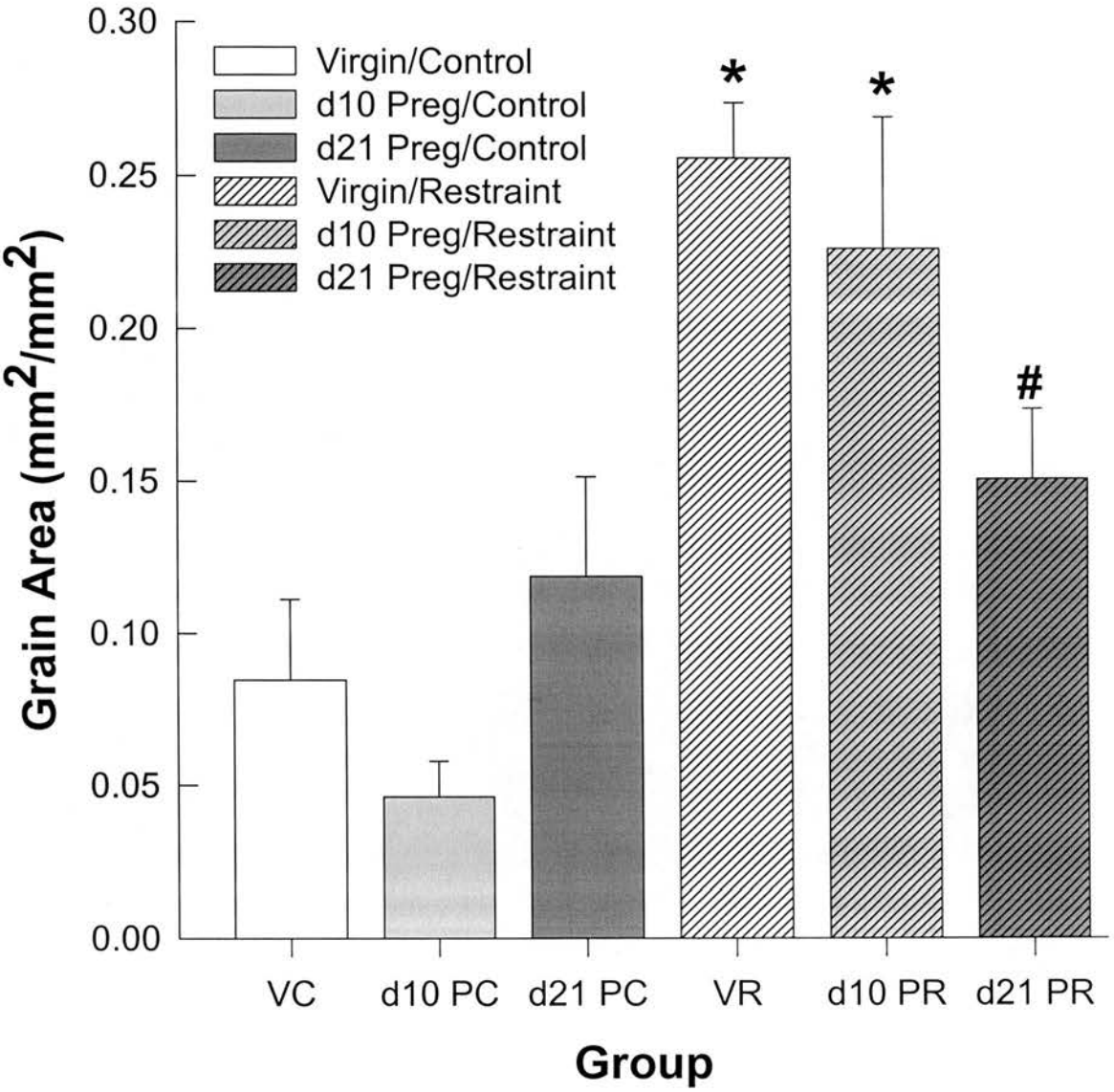
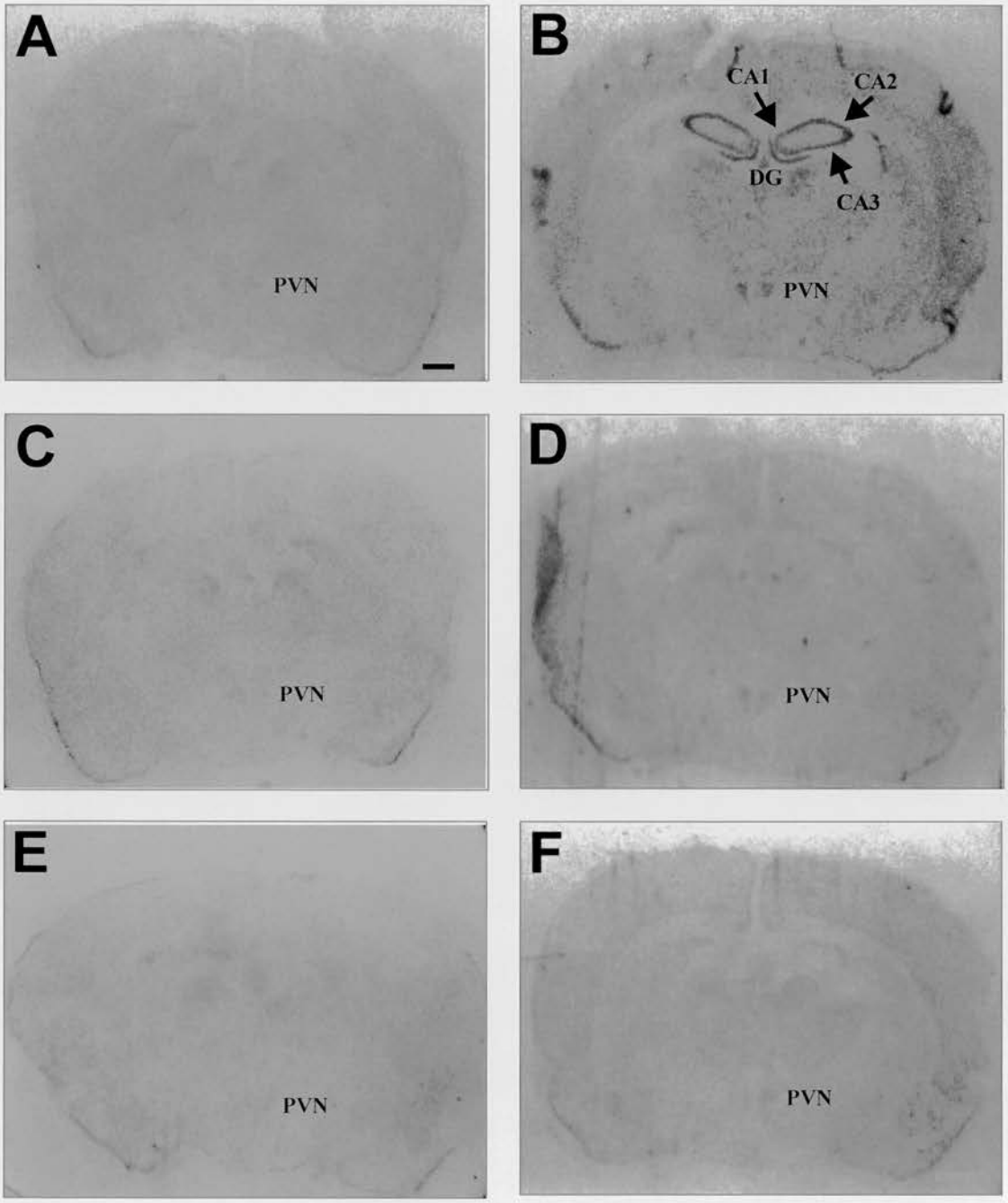


Figure 3.2.(a) The effect of 30 minutes restraint on NGFI-B mRNA expression in the pPVN of virgin and pregnant rats: Quantification of autoradiographs.

Rats were killed immediately after exposure to a 30 minute period of restraint. Coronal brain sections were hybridised with ^{35}S-labelled oligonucleotide probe for NGFI-B mRNA. Autoradiographs were quantified by computer based image analysis. Values plotted are group means ± SEM for grain area (mm^2/mm^2). Group numbers, as in figure 3.1. Two-way ANOVA followed by Student Newman-Keuls multiple comparison test was used for statistical analysis of the data: *$p < 0.001$ vs respective control group; #$p < 0.02$ vs virgin/restraint.



<u>Figure 3.2.(b)</u> The effect of 30 minutes restraint on NGFI-B mRNA expression in the pPVN of virgin and pregnant rats: Photomicrographs.

Coronal brain sections were hybridised with a ^{35}S-labelled oligonucleotide probe complementary to NGFI-B mRNA from A, virgin/control; B, virgin/restraint; C, day 10 pregnant/control; D, day 10 pregnant/restraint; E, day 21 pregnant/control; F, day 21 pregnant/restraint. PVN, paraventricular nucleus; CA1, CA2, CA3, hippocampal subfields; DG, dentate gyrus. Scale bar: 1mm.

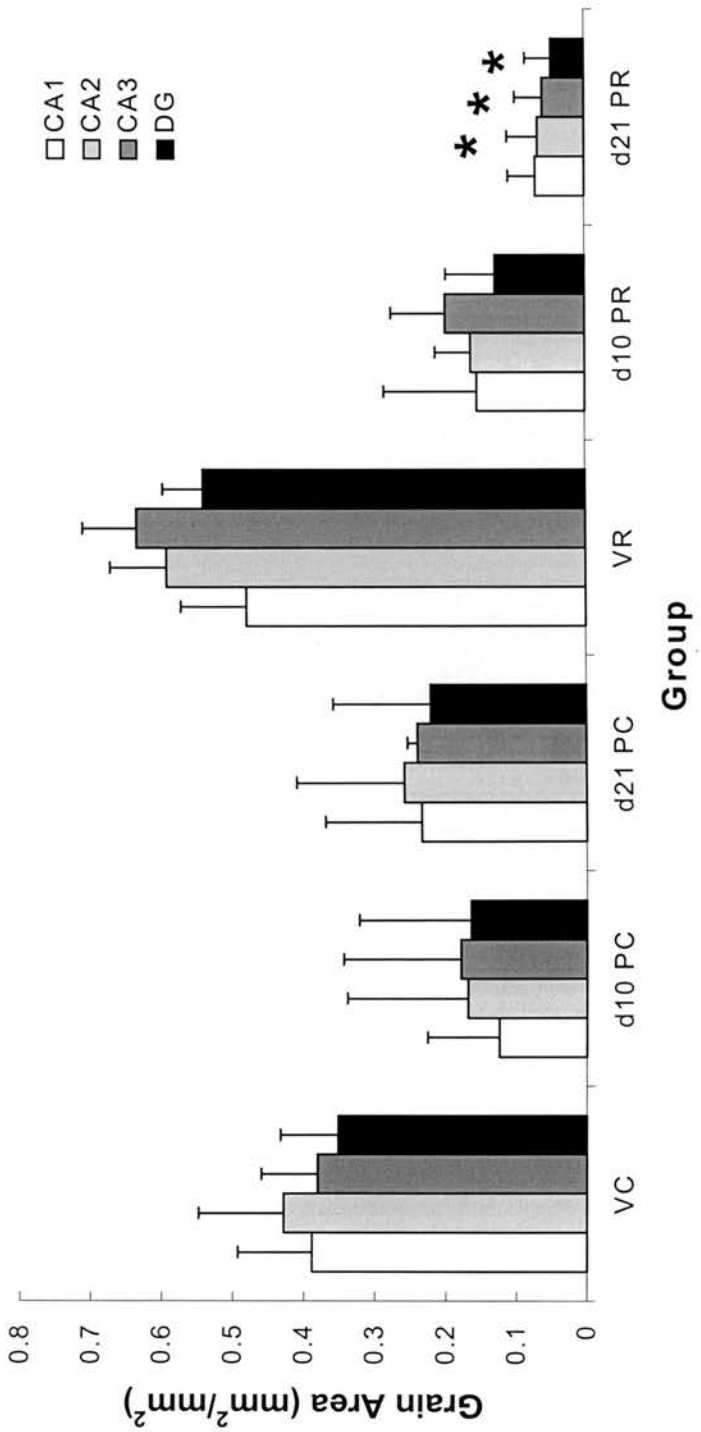


Figure 3.3. The effect of 30 minutes restraint on NGFI-B mRNA expression in the hippocampus of virgin and pregnant rats: Quantification of autoradiographs. Rats were killed immediately after exposure to a 30 minute period of restraint. Coronal brain sections were hybridised with a ^{35}S-labelled oligonucleotide probe for NGFI-B mRNA. Autoradiographs were quantified by computer based image analysis. Values plotted are group means ± SEM for grain area (mm^2/mm^2). Group numbers, as in figure 3.1. Abbreviations: VC, virgin/control; d10 PC, day 10 pregnant/control; d21 PC, day 21 pregnant/control; VR, virgin/restraint ; d10 PR, day 10 pregnant restraint; d21 PR, day 21 pregnant restraint; CA1, CA2, CA3, hippocampal subfields; DG, dentate gyrus. Two-way ANOVA followed by Student Newman-Keuls multiple comparison test was used for statistical analysis of the data: *$p < 0.05$ vs virgin/restraint group.

Experiment 2

3.3.4. Effects of restraint on AVP hnRNA expression in the pPVN

Analysis of film autoradiographs revealed AVP hnRNA expression in the pPVN was significantly less in the undisturbed pregnant rats than in the virgin group (two-way ANOVA, $p<0.02$; figure 3.4.). Restraint increased AVP hnRNA expression in the pPVN in the virgin but not in the day 21 pregnant rats (figure 3.4.). Levels of AVP hnRNA expression in the virgin restrained group were 600% of the virgin control group, whereas the pregnant restrained group level was 40% of that of the virgin control group.

Experiment 3

3.3.5. The effect of 30 minutes exposure to maternal defence on plasma ACTH and corticosterone levels in the intruders

Plasma concentrations of ACTH and corticosterone in the virgin and pregnant control rats did not differ (two-way ANOVA; figure 3.5.). Exposure to the maternal defence test caused a significant increase in plasma ACTH concentration in the virgin group but not in the pregnant group (figure 3.5). Thus the ACTH response in the virgin intruders (2.3-fold increase, $p < 0.001$) was significantly greater than in the pregnant intruders (1.4-fold increase, not significant). Similarly, the maternal defence test induced a significant increase in corticosterone secretion only in the virgin group and had no effect in the pregnant rats (figure 3.6.). In the virgin group plasma corticosterone was significantly elevated (36% increase) compared with the pregnant group (1.7% increase).

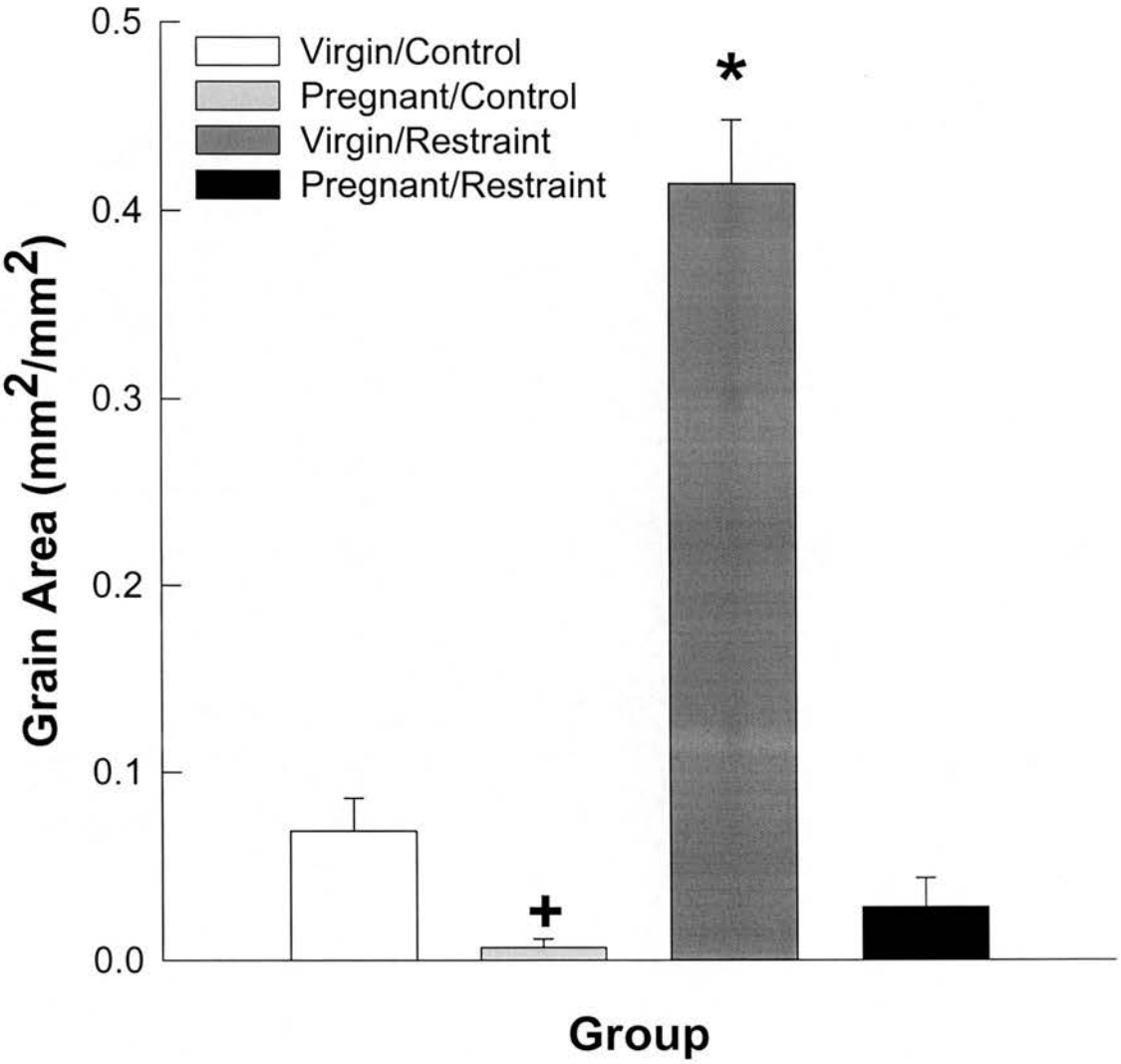
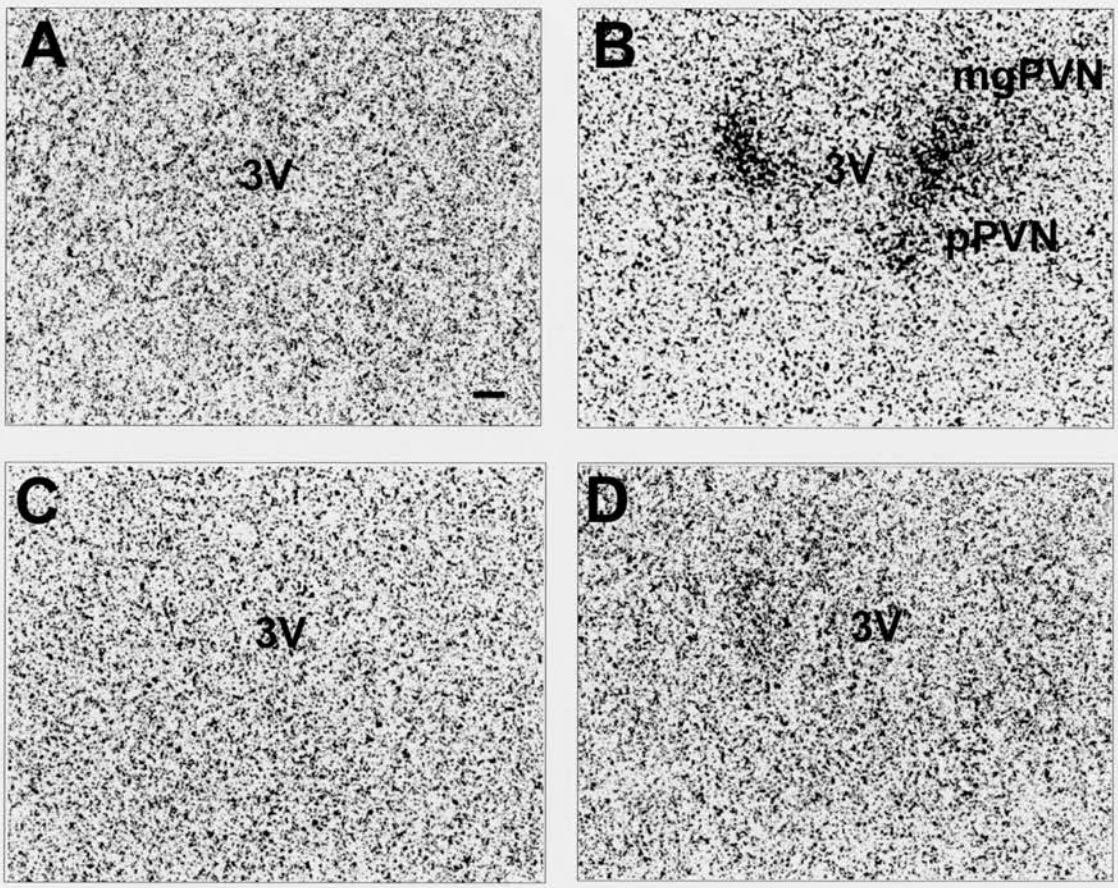


Figure 3.4.(a) The effect of 30 minutes restraint on AVP hnRNA expression in the pPVN of virgin and pregnant rats: Quantification of autoradiographs.

Rats were killed 90 minutes after the onset of exposure to a 30 minute period of restraint. Control rats were undisturbed prior to killing. Coronal brain sections were hybridised with ^{35}S-labelled oligonucleotide probe for AVP hnRNA. Autoradiographs were quantified by computer based image analysis. Values plotted are group means ± SEM for grain area (mm^2/mm^2). Group numbers: virgin/control, n=9; day 21 pregnant/control, n=7; virgin/restraint, n=6; day 21 pregnant/restraint, n=7. Two-way ANOVA followed by Student Newman-Keuls multiple comparison test was used for statistical analysis of the data: +$p < 0.02$ vs virgin/control; *$p < 0.001$ vs all other groups.



<u>Figure 3.4.(b)</u> The effect of 30 minutes restraint on AVP hnRNA expression in the pPVN of virgin and pregnant rats: Photomicrographs.

Brightfield photomicrographs of coronal sections through the paraventricular nucleus (PVN) hybridised with a ^{35}S-labelled oligo-probe complementary to arginine vasopressin heteronuclear RNA (AVP hnRNA) from A, virgin/control; B, virgin/restraint; C, day 21 pregnant/control; D=day 21 pregnant/restraint. pPVN, parvocellular division of paraventricular nucleus; mgPVN, magnocellular division of PVN; 3V, third ventricle. Scale bar: 100μm.

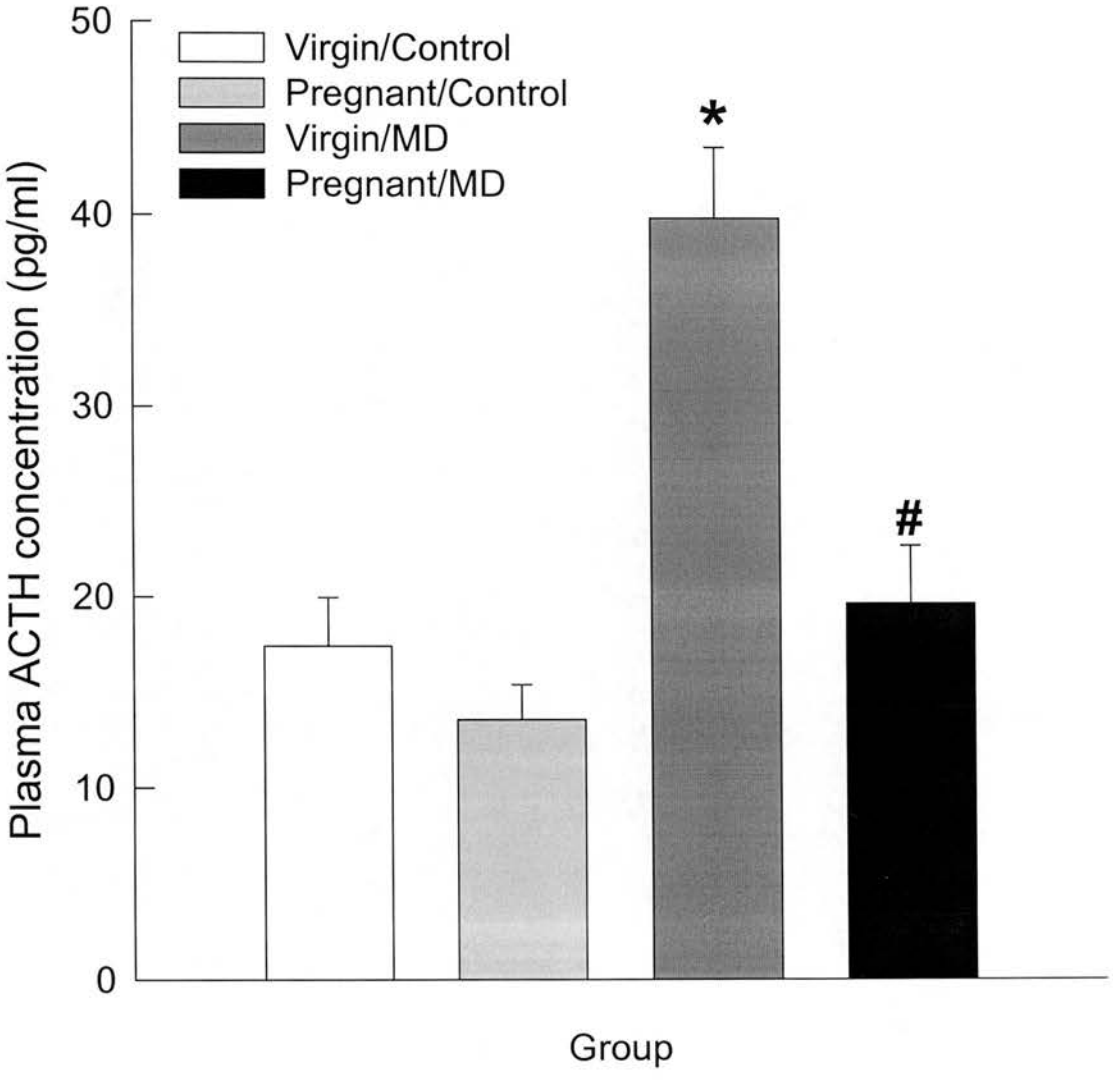


Figure 3.5. The effect of 30 minutes exposure to the maternal defence test on plasma ACTH concentrations in virgin and pregnant rats.

Trunk blood was collected immediately after exposure to the maternal defence (MD) test for 30 minutes. Control rats were undisturbed before being removed from their home cages and killed. Values are plotted as group means ± SEM. Group numbers: virgin/control, n = 5; pregnant/control, n=6; virgin/maternal defence, n = 8; pregnant/maternal defence, n = 5. Two-way ANOVA followed by Student Newman-Keuls multiple comparison test was used for statistical analysis of the data: *$p < 0.001$ vs virgin/control group; #$p < 0.001$ vs virgin/maternal defence group.

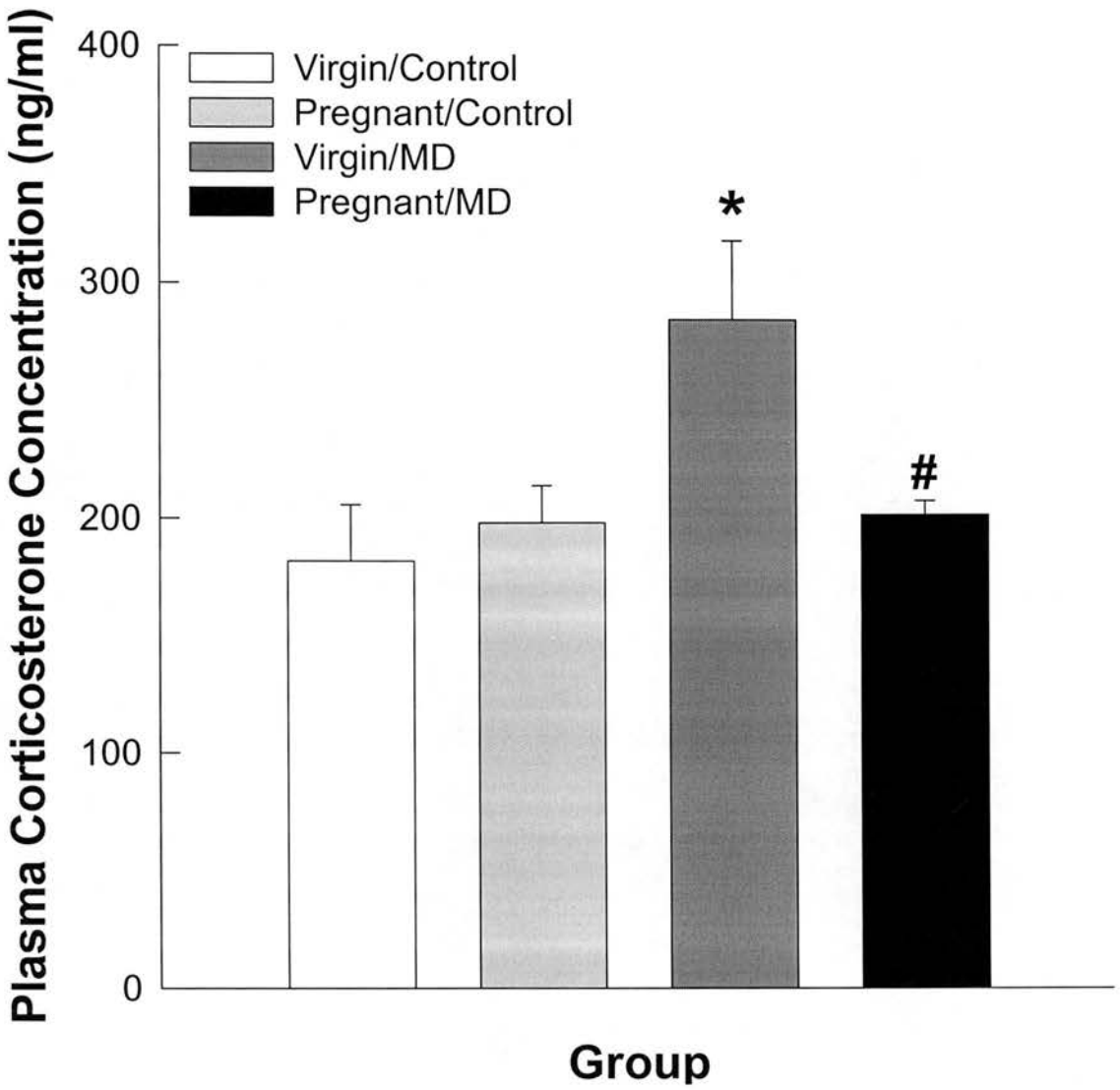


Figure 3.6. The effect of 30 minutes exposure to the maternal defence test on plasma corticosterone concentrations in virgin and pregnant rats.

Trunk blood was collected immediately after exposure to the maternal defence (MD) test for 30 minutes. Control rats were undisturbed before being removed from their home cages and killed. Values are plotted as group means ± SEM. Group numbers as before (see figure 3.5.). Two-way repeated measures ANOVA followed by Student Newman-Keuls multiple comparison test was used for statistical analysis of the data: *$p < 0.01$ vs virgin/control group; #$p < 0.03$ vs virgin/maternal defence group.

3.3.6. Behaviour of Lactating Resident

Aggressive behaviour displayed by the lactating resident was not significantly affected by the reproductive status of the intruder rat (table 3.1). Latency to attack a virgin (5.2 ± 3.0 minutes; t-test) or a pregnant intruder (3.5 ± 1.0 minutes; t-test) was not significantly different, nor was the number of attacks launched by the lactating residents (4.0 ± 0.7 vs 5.6 ± 1.0 against virgin and pregnant rats, respectively; t-test).

Reproductive status of intruder	Latency to attack (min)	Number of attacks
Virgin	5.2 ± 3.0	4.0 ± 0.7
Pregnant	3.5 ± 1.0	5.6 ± 1.0

Table 3.1. Aggressive behaviour displayed by the lactating resident against intruder rats. Aggressive behaviour displayed by the lactating resident was recorded throughout the 30 min maternal defence test. Specifically, latency to attack and the number of attacks launched were measured. Data is presented as group means ± SEM. Group numbers as before (see figure 3.5.). No significant differences were detected between any of the groups (Student's t-test; $p > 0.05$).

3.4. Discussion

Experiments 1 & 2 : Restraint Stress

The present data demonstrate that in rats the ACTH secretory response to restraint stress is attenuated in late pregnancy (day 21). This confirms previous studies employing other stressors (eg. elevated plus maze and forced swimming) in late pregnancy (Neumann *et al* , 1998). Following exposure to restraint stress, plasma ACTH concentration in the day 10 pregnant rats was not different from that of the virgin rats, however in late pregnant rats the ACTH response was significantly reduced. This suggests that the attenuated ACTH response to stress is manifested in mid-gestation, at sometime after day 10 of pregnancy. These results are consistent with previous studies using other stressors (Neumann *et al* , 1998).

Restraint induced an increase in pPVN NGFI-B mRNA expression in all of the groups, however the level of expression in the day 21 pregnant group was significantly attenuated when compared with the virgin and day 10 pregnant group. The decreased NGFI-B mRNA response to restraint in the pPVN of the late pregnant rats indicates that the parvocellular neurones in the PVN are stimulated less strongly by the stressor. The blunted ACTH response observed in the day 21 pregnant group is likely to be a result of reduced neuronal activation in the pPVN, indicated by the attenuated NGFI-B response. Since NGFI-B is a putative regulator of CRH gene transcription (Wilson *et al* , 1991), the results suggest that restraint is less effective in activating CRH gene transcription in late pregnant rats. In the hippocampus, stimulation of NGFI-B mRNA expression by restraint stress was also reduced in the day 21 pregnant group indicating reduced activation of stressor processing circuits in pregnancy (Herman & Cullinan, 1997). These results are consistent with those reported by Da Costa and colleagues, who demonstrated reduced *c-fos* mRNA expression in the hippocampal subfields following central administration of CRH in lactating rats compared with virgin rats (da Costa *et al* , 1997).

Restraint is a good example of an emotional (or neurogenic) stressor. Emotional stressors often rely upon somatosensory pathways for their transduction and involve a distinct cognitive and/or affective component. These stressors have common features, they require processing and integration from sensory modalities and they do not involve an immediate threat, but rather constitute stimuli that become stressful only by comparison with previous experiences. HPA axis responses to restraint stress involve processing by the limbic system (Sawchenko *et al*, 2000). As well as in the PVN, restraint stress induces Fos expression in the prefrontal cortex, central (CeA) and medial amygdaloid (MeA) nuclei, bed nucleus of the stria terminalis (BNST), lateral septum, hippocampus, median preoptic area (MPOA), dorso-medial hypothalamus, locus coeruleus (LC) and in brainstem noradrenergic (A1 and A2) and adrenergic (C1 and C2) nuclei (Melia *et al*, 1994; Herman & Cullinan, 1997; Chowdhury *et al*, 2000; Sawchenko *et al* , 2000; Dayas *et al*, 2001). Lesions to the prefrontal cortex, hippocampus, septum or amygdala result in a reduced HPA axis response to restraint (Herman & Cullinan, 1997). In particular, the MeA appears to play an important role in activation of the HPA axis in response to restraint stress. Some of the MeA neurones activated following restraint have been shown to project directly to the PVN and ibotenic lesions of the MeA, but not the CeA greatly reduce restraint-induced activation of the pPVN neurones (Dayas *et al*, 1999). In pregnancy, processing of stress stimuli through the MeA (or any of the other aforementioned brain regions) may be disrupted thus explaining reduced activation of the pPVN neurones and hence the HPA axis.

Generally the hippocampus is thought of as having an inhibitory effect on the HPA axis (Herman *et al*, 1989; Herman *et al*, 1992). However, hippocampal regulation of the HPA axis is complex and lesions can result in an increase or a decrease in glucocorticoid secretion and electrical stimulation of the hippocampus can stimulate or inhibit corticosterone secretion (Dunn & Orr, 1984). It is not clear from these experiments whether the hippocampal neurones are exerting an overall negative or positive effect on HPA activity. If these neurones are exerting an excitatory influence over the HPA axis then it follows that reduced activation of the hippocampal subfields in response to restraint in the late pregnant rats would correlate with

reduced HPA axis activation. If on the other hand these neurones are exerting a net inhibitory influence over HPA activity then it is possible that in pregnancy the pPVN CRH neurones overcome this reduced inhibitory drive from the hippocampus and remain less responsive. This may also explain the more prolonged ACTH secretory response to stress (although still markedly less than in virgins) frequently observed in pregnancy (Douglas *et al*, 1998; Neumann *et al* , 1998) associated with a loss of glucocorticoid feedback inhibition of the HPA axis.

Basal expression of AVP hnRNA in the pPVN was significantly less in the day 21 pregnant group compared with the virgin group. This is consistent with findings indicating reduced CRH and AVP mRNA expression at the end of pregnancy (Johnstone *et al* , 2000a). Exposure to restraint stress significantly increased AVP hnRNA expression in the pPVN of virgin rats, which is in agreement with previous studies using this and other stressors (Kovacs & Sawchenko, 1996; Ma *et al* , 1997). The absence of an increase in AVP hnRNA expression in the pPVN in late pregnant rats further suggests reduced activation of CRH/AVP pPVN neurones by restraint stress in pregnancy. The promoter region of the AVP gene contains two functional cAMP and a putative NGFI-B response element, as well as an AP-1 binding site (Kovacs & Sawchenko, 1996). Either of these may play a role in translating stress-related input into AVP gene transcription. Thus reduced expression of NGFI-B mRNA in the pPVN of pregnant rats following exposure to restraint (as reported in experiment 1) may lead to reduced activation of the AVP gene in response to restraint in pregnancy (as described in experiment 2). Alternatively, changes in AVP gene transcription in response to stress in pregnancy may be the result of changes in CREB phosphorylation (Kovacs & Sawchenko, 1996).

Together these data suggest that reduced stimulation of ACTH (and thus corticosterone) secretion by an emotional stressor (restraint) in pregnancy is a consequence of reduced stimulation of CRH/AVP neurones in the parvocellular region of the PVN. The hippocampus may be involved in the circuitry regulating these neurones, and stress-induced activation of the neurones here is also less in the day 21 pregnant rats compared with the virgin rats. The causes of these changes are

unclear. The CRH/AVP neurones themselves may be less responsive to stressful stimuli in late pregnancy or it may be that the neurones impinging upon the pPVN neurones are less activated by stressors and thus the central drive to these neurones is reduced in pregnancy. Thus, the adaptations that occur at the level of the anterior pituitary in pregnancy (Neumann *et al* , 1998) are likely to be a consequence of reduced drive from the CRH/AVP neurones, rather than the primary cause of the attenuated responsiveness of the HPA axis in pregnancy.

Experiment 3: Maternal Defence Test

To meet the concerns regarding the 'physiological relevance' of restraint stress to a late pregnant rat, exposure to the maternal defence test was used in experiment three. Exposure to a maternally aggressive lactating resident for 30 minutes (which involves olfactory, visual and auditory cues) significantly activated the HPA axis in the virgin intruders; this is reflected by increased levels of plasma ACTH and corticosterone concentration in the trunk blood. Comparison of the hormonal responses of the virgin and pregnant intruders revealed that the maternal defence test failed to evoke a significant ACTH or corticosterone response in the day 21 pregnant group, confirming previous results demonstrating blunted ACTH and corticosterone to the elevated plus maze and forced swimming (Neumann *et al* , 1998). Furthermore these results are consistent with the attenuated ACTH response to restraint in late pregnant rats, described in experiment 1 of this chapter. The differences in the ACTH and corticosterone secretory responses following the maternal defence test are not a consequence of differential behaviour displayed by the resident dependent on the reproductive status of the intruder, since a similar number of attacks were launched by the resident whether the intruder was a virgin or pregnant. The brain pathways involved in processing stimuli to effect an HPA response to maternal aggression are not known though probably involve limbic forebrain pathways, as is the case with other emotional stressors (Herman & Cullinan, 1997). Fos expression is induced in the lateral septum, bed nucleus of stria terminalis, lateral preoptic area, lateral hypothalamic area, paraventricular nucleus, medial and central amygdala as well as in the autonomic and monoaminergic nuclei of the brainstem in male rats following social defeat (Martinez *et al*, 1998). It is likely that the pathways involved in female

rats are similar to those in males. Thus any interruption in the processing of these stimuli by the various forebrain and brainstem structures in pregnancy may result in reduced activation of the HPA axis.

In conclusion, as with forced swimming and the elevated plus maze (Douglas *et al* , 1998; Neumann *et al* , 1998), late pregnant rats display reduced HPA axis responses to the emotional stressors, restraint and maternal aggression. In the case of restraint this appears to be a result of reduced drive by CRH and/or AVP, as indicated by reduced activation of the pPVN neurones.

To date no study has investigated whether pregnant rats display attenuated HPA axis responses to *physical* stressors. In the next chapter the effects of immune challenge on HPA axis activation are investigated in late pregnancy.

CHAPTER 4

Effects of physical stressors on the hypothalamo-pituitary-adrenal axis in pregnancy

4.1. Introduction

Following immune activation an organism elicits a physiological response during which information is conveyed to the brain to enable the organism to make the appropriate endocrine and behavioural adaptations necessary to restore physiological balance. As mentioned in Chapter 1, an important interaction occurs between the neuroendocrine and immune axes and it is now widely accepted that activation of the HPA axis plays a vital role in restoring homeostasis following immune challenge (Turnbull & Rivier, 1995).

In Chapter 3, the responsiveness of the HPA axis to emotional (or psychological) stressors was shown to be markedly reduced in pregnancy. To determine whether a mechanism is in place in late pregnancy to restrain the responsiveness of the HPA axis to all stressors, it was important to investigate HPA responses to a 'purely' physical stressor. To date, no previous studies have investigated the effects of immune challenge (a physical stressor) on HPA activity in late pregnancy.

4.1.1. Effects of endotoxin LPS and cytokines on the HPA axis

In non-pregnant animals various models have been used to investigate the HPA response to infection, inflammation and tissue damage. Peripheral administration of endotoxin lipopolysaccharide (LPS) is one such model. LPS is a component of the cell wall of gram-negative bacteria such as Escherichia coli (Aubry *et al*, 1997). Acute administration of LPS acts as a potent stimulator of the HPA axis (Takemura *et al*, 1997; Turnbull *et al*, 1998). Systemic LPS injection increases ACTH and corticosterone secretion (Turnbull *et al* , 1998), CRH release (Givalois *et al*, 1995) and CRH mRNA expression in the PVN (Kakucska *et al*, 1993) and is associated with an increase in the circulating levels of cytokines (Andersson *et al*,), such as tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) released from activated macrophage cells. The mechanism(s) by which LPS activates the HPA axis is not clear but cytokine mediated interactions with the hypothalamus

(Lenczowski *et al*, 1997) and activation of vagal inputs to the CNS (Gaykema *et al*, 1995b; Kapas *et al*, 1998) are thought to be important. Cytokines such as TNF-α, IL-1 and IL-6 have all previously been shown to activate the HPA axis after systemic administration and antisera to these cytokines significantly attenuate the HPA responses to LPS endotoxin (Besedovsky & Del Rey, 1992). Furthermore passive immunisation of IL-1 significantly attenuates the response of the HPA axis to LPS in rodents (Besedovsky & Del Rey, 1992).

LPS evokes secretion of cytokines from circulating monocytes/neutrophils. In order to do this, the endotoxin must reach the bloodstream where it binds with LPS-binding protein (LBP). This complex then binds to the membrane CD14 receptor located on the cell surface of phagocytes and triggers the release of cytokines (Wright *et al*, 1990). Recently it has been reported that LPS induced the expression of CD14 mRNA in circumventricular organs (Lacroix *et al*, 1998) (brain structures which lack an effective blood-brain-barrier) suggesting that LPS can possibly have a direct action on the brain, rather than being dependent upon the production of cytokines in the periphery.

Systemic administration of IL-1β is a potent CRH secretagogue (Harbuz *et al*, 1992; Besedovsky & Del Rey, 1992), leading to increased ACTH secretion from the anterior pituitary and consequently elevated plasma concentrations of corticosterone. CRH plays an important role in mediating IL-1β-induced ACTH release. It has been shown that immuno-neutralisation of endogenous CRH inhibits IL-1β-induced ACTH secretion (Sapolsky *et al*, 1987; Berkenbosch *et al*, 1987) and PVN lesions attenuate the ACTH secretory response to IL-1β administration (Rivest & Rivier, 1991).

4.1.2. How do cytokines activate the HPA axis?

As discussed in the General Introduction Chapter, the precise mechanisms and sites of action through which cytokines interact with the HPA axis are unclear, however a strong body of evidence supports the theory that IL-1 acts on the endothelium of brain blood vessels to activate the release of intermediate mediators, such as prostaglandins (see section 1.17. of Chapter 1). Indeed brain microvessels express the IL-1 type 1 receptor (Ericsson *et al*, 1995). The synthesis of prostaglandins (PG) is initiated by the action of cyclooxygenase (COX). Intravenous administration of LPS and IL-1β induces COX-2 mRNA and protein along blood vessels of the entire brain microvasculature, choroid plexus and meninges. Furthermore, administration of COX inhibitors (e.g. ibuprofen and indomethacin) almost completely abolishes the HPA axis response to IL-1β administration (Katsuura *et al*, 1988; Watanabe *et al*, 1990; Morimoto *et al*, 1991; Rivier, 1993; Niimi *et al*, 1997).

It is generally held that brainstem catecholamine cell groups in the nucleus of the solitary tract (NTS) and ventro-lateral medulla (VLM), (in particular the VLM A1 and NTS A2 noradrenergic neurones) are likely to be important sites for receiving and integrating peripheral immune signals and conveying this information to the CRH neurones in the PVN (see figure 4.1.). It has previously been shown that brainstem catecholamine cells activated by IL-1β project to the PVN (Ericsson *et al*, 1994). Furthermore transection of ascending catecholamine projections from the brainstem to the hypothalamus (Ericsson *et al* , 1994), destruction of PVN catecholaminergic terminals (Weidenfeld *et al*, 1989) and lesions to the NTS and VLM (Buller *et al*, 2001) all result in attenuated IL-1β-induced HPA axis responses. The PG receptor, EP3 is expressed in the NTS and VLM neurones, which may represent a mechanism by which peripheral immune signals activate these brainstem neurones.

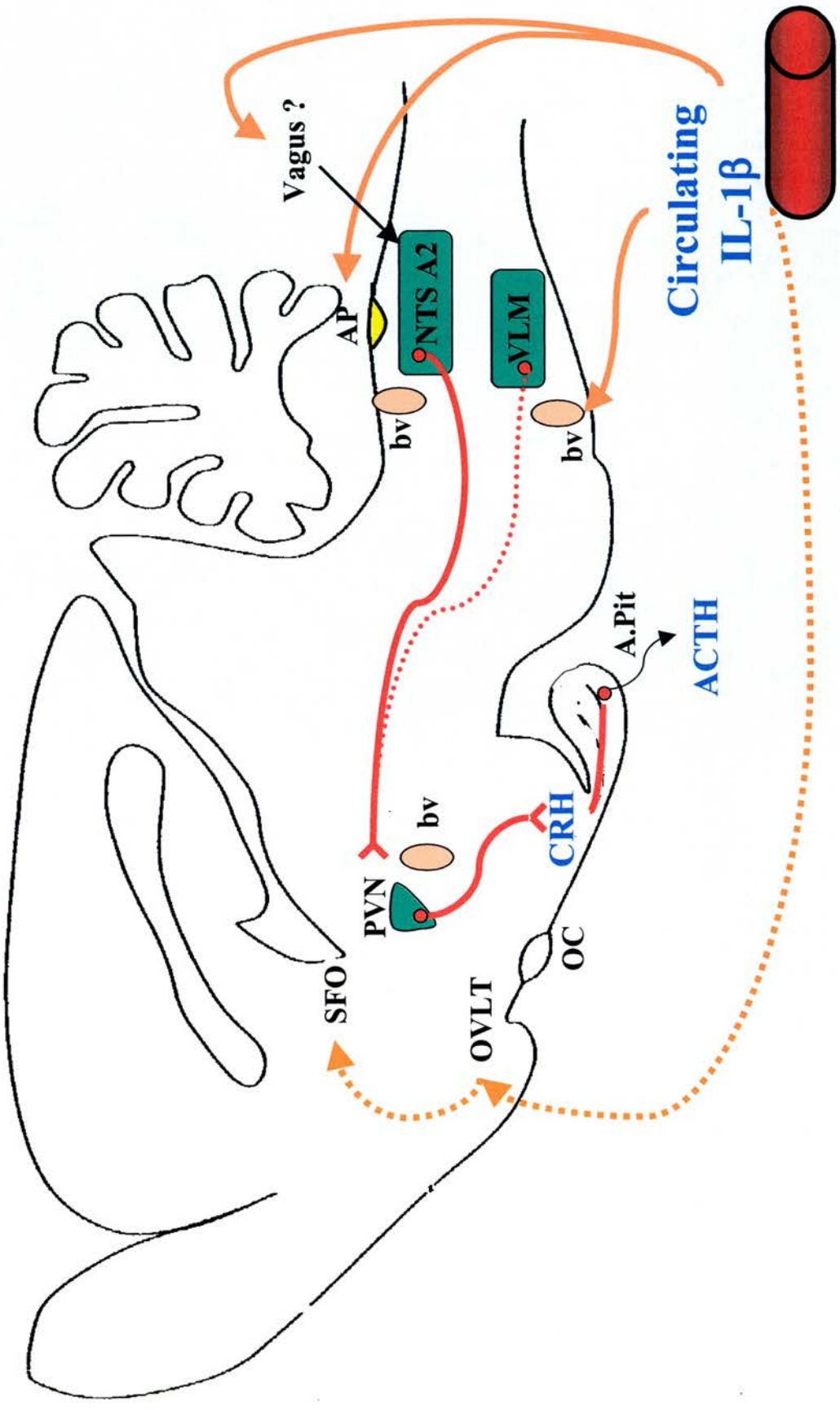

Vagus ?
AP
NTS A2
VLM
bv
bv
Circulating
IL-1β
A.Pit
ACTH
bv
CRH
PVN
SFO
OVLT
OC

Figure 4.1. Possible pathways involved in IL-1β-induced activation of the HPA axis

Illustration showing the possible circuitry mediating the activation of the PVN neurones and hence the HPA axis following administration of IL-1β. The circumventricular organs and the blood vessels (bv) are important target sites of cytokines of systemic origin. The nucleus of the solitary tract (NTS) and the ventrolateral medulla (VLM) in the brainstem play a role in integrating information from the periphery. Abbreviations: AP, area postrema; A.Pit, anterior pituitary; ACTH, adrenocorticotropic hormone; CRH, corticotropin releasing hormone; OC, optic chiasm; OVLT, organum vasculosum of the lamina terminalis; PVN, paraventricular nucleus; SFO, subfornical organ.

Thus brainstem catecholamine neurones seem to play an important role in generating HPA responses to systemic administration of IL-1β. However, in addition to the direct projections from the brainstem to the pPVN, indirect pathways via forebrain structures may also be involved in mediating activation of the CRH neurones in response to systemic IL-1β. In particular the central nucleus of the amygdala (CeA) is a possible site important in regulating HPA axis responses to systemic IL-1β. The concept that the CeA is involved in modulating HPA activity was realised some time ago. Electrical stimulation of the CeA causes an increase in ACTH release, whilst lesions of the CeA attenuate HPA axis responses to a wide variety of stimuli (Beaulieu *et al*, 1986; Beaulieu *et al*, 1987; Feldman *et al*, 1994). Ericsson and colleagues (Ericsson *et al* , 1994) first demonstrated that neurones in the CeA are robustly activated in response to systemic IL-1β (Ericsson *et al* , 1994; Buller *et al*, 1998; Xu *et al*, 1999). However more recently it has been shown that bilateral ibotenic acid lesions of the CeA causes a significant reduction in the number of PVN CRH cells activated and a significant attenuation of the ACTH secretory response to i.v. IL-1β (Xu *et al* , 1999).

The number of direct neural projections from the CeA to the pPVN is restricted to only a small population of cells and it has been reported that CeA neurones activated (as demonstrated by Fos expression) by IL-1β are distinct from those found to project directly to the PVN (Ericsson *et al* , 1994). Thus it seems likely that the CeA influences CRH neurone activity via indirect neural pathways. A potential 'relay centre' is the bed nucleus of the stria terminalis (BNST). Anatomical studies have demonstrated a direct connection between the CeA and BNST (Sun *et al*, 1991). *C-fos* mRNA expression in the BNST is increased following systemic IL-1β administration (Ericsson *et al* , 1994; Buller *et al* , 1998) and CeA lesions significantly reduce the levels of IL-1β-induced *c-fos* expression in the BNST (Xu *et al* , 1999). Noradrenergic neurones in the VLM (A1) and NTS (A2) have been shown to project to the CeA (Jia *et al*, 1992; Roder & Ciriello, 1993; Roder & Ciriello, 1994; Zardetto-Smith & Gray, 1995). It has been reported that NTS (but not VLM) ibotenic acid lesions significantly reduce the number of CeA cells activated in response to IL-β (Buller *et al* , 2001), thus indicating the importance of these

brainstem populations in conveying information to the CeA following immune insult via IL-1β.

The mechanism by which LPS/cytokines activate the HPA axis seems dependent upon the route of administration. Vagal afferents play an important role in effecting HPA responses to immune challenge (LPS and IL-1β) following administration via the intra-peritoneal route (Fleshner *et al*, 1995). Vagal lesions block ACTH responses to i.p. LPS and IL-1β and inhibit *c-fos* mRNA expression in the pPVN and NTS and inhibit NA release in the PVN (Gaykema *et al*, 1995a; Fleshner *et al*, 1997; Hansen *et al*, 1998), normally evoked following immune challenge. However, vagotomy does not significantly affect these responses when LPS and IL-1β are administered by other routes, e.g. intravenously. Activation of vagal afferents by i.p. LPS and IL-1β appears to be at least partly dependent upon the production of PGs at the vagal somata (Ek *et al*, 1998). Therefore it seems that the participation of vagal sensory mechanisms in mediating the HPA response to immune challenge is limited to i.p. delivery of LPS/cytokine or local peritoneal inflammation.

In summary, peripherally administered IL-1β acts on type 1 IL-1 receptors located on or closely associated with blood vessels at circumventricular organs (one possibility is the area postrema). This results in the local production of prostaglandins (via COX pathways), which are soluble and may diffuse through brain parenchyma to activate brainstem catecholaminergic neurones (see figure 4.1.). These activate PVN CRH neurones via direct and/or indirect projections (via the CeA and the BNST).

The aim of the present experiments was to determine whether pregnant rats demonstrate reduced HPA axis responses to immune challenge, and if so to investigate some of the underlying mechanisms. Immune challenge with i.v. administration of either LPS or IL-1β was employed as a physical stressor. There are several advantages to using immune challenge, firstly LPS/IL-1β can be administered i.v. via a previously implanted cannula, thus minimising disturbance to the animal and preventing the introduction of non-specific emotional stressors through handling and removing the rat from its home cage. Secondly, activation of

the HPA axis by immune challenge does not rely on the rat's cognitive function or perception of the stressor, and finally IL-1β acts, at least in part, via a well-defined monosynaptic pathway.

Noradrenergic inputs to the PVN appear to be important in mediating HPA responses to immune challenge. In experiment 2, Fos activation was measured in the A2 cell group (including noradrenaline cells) of the NTS in the brainstem, to establish if changes here may explain the hyporesponsive nature of the HPA axis to IL-1β in pregnancy.

Oxytocin is a so-called 'stress hormone' in the rat (Lang *et al*, 1983). Previous studies have shown that oxytocin secretion following exposure to forced swimming is modestly attenuated in late pregnancy. This seems to be a result of enhanced opioid inhibition of oxytocin neurones (Douglas *et al*, 1998), as treatment with the opioid receptor antagonist naloxone, prior to forced swimming, reveals a greatly enhanced oxytocin secretory response (compared to the virgin group). In the present experiments plasma oxytocin was measured to establish whether this is affected by systemic IL-1β. Since endogenous opioids have been shown to restrain oxytocin secretion in response to stress in late pregnancy, it seemed plausible that they may also be involved in restraining HPA activity. Previously Douglas *et al* (Douglas *et al* , 1998) have shown that treatment with naloxone reduces the ACTH secretory response to forced swimming in female virgin rats, whilst causing a modest increase in ACTH secretion and restoring a corticosterone secretory response in pregnant rats. Thus the effects of removing opioid inhibition on the HPA axis and the neurohypophysial oxytocin system in response to immune challenge were measured.

The aims of this set of experiments were:

(i) to establish whether the HPA axis and oxytocin system was less responsive to stimulation with LPS and IL-1β in late pregnancy, and if so

(ii) to establish whether this is a consequence of differential activation of the brainstem NTS neurones in response to IL-1β in pregnancy.

(iii) and investigate a role for endogenous opioids in restraining HPA axis and oxytocin secretory responses to immune challenge in pregnancy.

4.2. Materials and Methods

4.2.1. Animals

Female Sprague Dawley rats were used throughout this set of experiments and were maintained under conditions detailed in chapter 2. After surgery all rats were caged individually.

4.2.2 Surgery

Five days prior to the day of the experiment, virgin and pregnant rats (day 16 of pregnancy) were fitted with a jugular vein cannula under inhalation of halothane anaesthesia, as described previously (see section 2.3. of chapter 2).

4.2.3. Experimental Procedure

Blood Sampling

For blood sampling experiments rats had the jugular vein cannula connected to PVC extension tubing (wall = 1mm, internal diameter = 0.5mm) filled with heparinised saline (1ml heparin; 5000 units/ml in 100ml 0.9% saline) and attached to a 1ml syringe, on the morning of the experiment (between 07:30-09:00h). Rats were then left undisturbed for at least one hour prior to the start of blood sampling.

The volume of blood withdrawn was dependent upon the eventual assay. For analysis of oxytocin, 0.3 ml samples were taken, for ACTH, 0.5 ml and for ACTH and corticosterone 0.6 ml samples were taken. Blood samples taken for analysis of ACTH or corticosterone were collected into 1 ml syringes containing 5% EDTA and those taken for oxytocin analysis were collected in syringes containing heparinised saline (see general methods; chapter 2). In each case blood was stored in eppendorfs on ice until centrifugation (see general methods). The blood sampling protocol used was different for each experiment due to different action times of LPS and IL-1β on the HPA axis and hypothalamo-neurohypophysial system. Details of the precise protocol used are given below.

Experiment 1:

Two basal blood samples (for ACTH) were taken 60 min apart. Following the second basal sample, rats were administered either 1μg/kg lipopolysaccharide (LPS) endotoxin (2 μg/ml) or 0.9% saline i.v. Four more blood samples were then taken 60, 120, 180 and 240 min after the injection.

Experiment 2:

Two basal blood samples were taken 30 min apart. Following the second basal blood sample, rats were treated i.v. with either 500 ng/kg human recombinant (rh) IL-1β (1 μg/ml) or the appropriate volume of vehicle (0.2 % bovine serum albumin in phosphate buffered saline; 0.2% BSA in PBS). Sequential blood samples were withdrawn 15, 30, 60, 90 and 120 min after the injection. Rats were killed by conscious decapitation 4h after administration of the IL-1β/vehicle. Trunk blood was collected into universal tubes containing chilled 5% EDTA solution. After centrifugation (see General Methods chapter), plasma was separated and stored at -20°C until radioimmunoassay for ACTH, corticosterone and oxytocin. Brains and brainstems were rapidly removed and frozen on dry ice for CRH mRNA *in situ* hybridisation and Fos immunocytochemistry, respectively.

A separate group of rats was fitted with jugular cannulae as before. On the day of the experiment, jugular vein cannulae were connected to extension tubing and animals were left undisturbed for one hour. Each rat was then treated i.v. with either 500 ng/kg rh IL-1β (1 μg/ml) or vehicle (0.2% BSA in PBS). Ninety minutes after the injection (the optimum time for Fos protein expression) rats were killed by decapitation. Brainstems were rapidly removed and frozen on dry ice, as before. Brainstems were then transferred to a -70°C freezer until sectioning and Fos immunocytochemistry.

Experiment 3:

One basal blood sample was taken (for ACTH). Immediately after the first blood sample, rats were treated with either 5 mg/kg naloxone (10 mg/ml) or an equivalent volume of 0.9 % saline, intravenously. One post-naloxone/vehicle blood sample was withdrawn 15 minutes after the injection. All rats were then treatedadministered i.v. with 500 ng/kg rh IL-1β (1 μg/ml). Further blood samples (all for ACTH) were taken 15, 30, 60 and 90 min after administration of IL-1β.

Experiment 4:

One basal blood sample was taken (for oxytocin). Immediately after the first blood sample, rats were treated with either 5 mg/kg naloxone (10 mg/ml) or vehicle (0.95 saline) i.v. Two post-naloxone/vehicle blood samples were withdrawn 5 and 15 min after the injection. All rats were then treated with 500 ng/kg rh IL-1β (1 μg/ml) i.v. Further blood samples (all for oxytocin) were then taken 5, 15, 30 and 60 min after administration of IL-1β. Rats were killed by decapitation 4 hours after the IL-1β injection. Brains were rapidly removed (for CRH mRNA *in situ* hybridisation) and frozen on dry ice, as before.

4.2.4. *In situ* hybridisation

Brains for *in situ* hybridisation were sectioned coronally at 15μm and mounted on gelatinised slides as previously described in the General Methods chapter. To detect

CRH mRNA expression a 42-mer oligonucleotide probe was used [MWG-Biotech]. The sequence of the CRH mRNA oligo-probe used is given below. It is complementary to bases 496-537 which encode amino acids 22-35 of the rat CRH peptide (Jingami *et al*, 1985).

5'- CCT GTT GCT GTG AGC TTG CTG AGC TAA CTG CTC TGC CCT GCC-3'

Probe labelling and hybridisation were performed as described in the General Methods chapter. The post-hybridisation washes were performed in the usual way.

The melting temperature of the CRH mRNA oligo-probe is 78°C, therefore the heated SSC washes were performed at 58°C. Once dry, the sections were exposed to autoradiographic film for 21 days at room temperature. CRH mRNA expression in the PVN was quantified from autoradiographs using a computer based image analysis system as described in the General Methods Chapter.

4.2.5. Fos Immunocytochemistry

Brainstem sections (15μm) were cut through the A2 cell region of the nucleus of the solitary tract (at the level of the area postrema) on a cryostat and mounted on gelatinised slides. Fos immunocytochemistry was performed using the method described in section 2.5.1. of the General Methods chapter. Fos positive stained cells were counted in the NTS in 8-10 sections from each rat. The area of the NTS was measured using an AppleMac computer and NIH Image software.

4.2.6. Radioimmunoassays

All samples from a particular experiment were always assayed together. Plasma ACTH and corticosterone concentrations were determined using commercially available kits (see sections 2.6.1. and 2.7.). The sensitivity was 1 pg/ml and 0.4 ng/ml, respectively and the intra-assay variation was <11% and <6%, for the ACTH and corticosterone assays respectively. Plasma oxytocin concentration was

determined using an in-house assay based on a method described by Higuchi *et al* (Higuchi *et al*, 1985). The sensitivity of the assay was 4.4 pg/ml and the intra-assay variation <12%.

4.2.7. Statistical Analysis

Two way repeated measures analysis of variance (2-way RM ANOVA), followed by Student-Newman-Keuls multiple comparison test was used to analyse plasma ACTH, corticosterone and oxytocin data. CRH mRNA and Fos data were analysed using a two-way ANOVA. In each case, p values less than 0.05 were considered statistically significant.

4.3. Results

Experiment 1

4.3.1. Effects of i.v. LPS on plasma ACTH concentration

Basal plasma concentrations of ACTH were not different between any of the groups. Administration of isotonic saline had no significant effect on plasma ACTH in either the virgin or the pregnant group (figure 4.2.). Intravenous LPS induced a significant increase in plasma ACTH concentration in the virgin group ($p<0.001$; 2-way RM ANOVA), which was significantly higher than basal levels within 60 min (70-fold increase) of the injection, peaked 120 min (85-fold increase) after the injection, and was still significantly elevated 240 min after LPS administration. However, LPS evoked only a small increase in plasma ACTH concentration in the pregnant group, with levels not significantly changed from basal levels (figure 4.2). ACTH secretion peaked 120 min after the LPS injection and was 137.8 ± 6.9 pg/ml in the virgin group and 28 ± 8.9 pg/ml in the pregnant group.

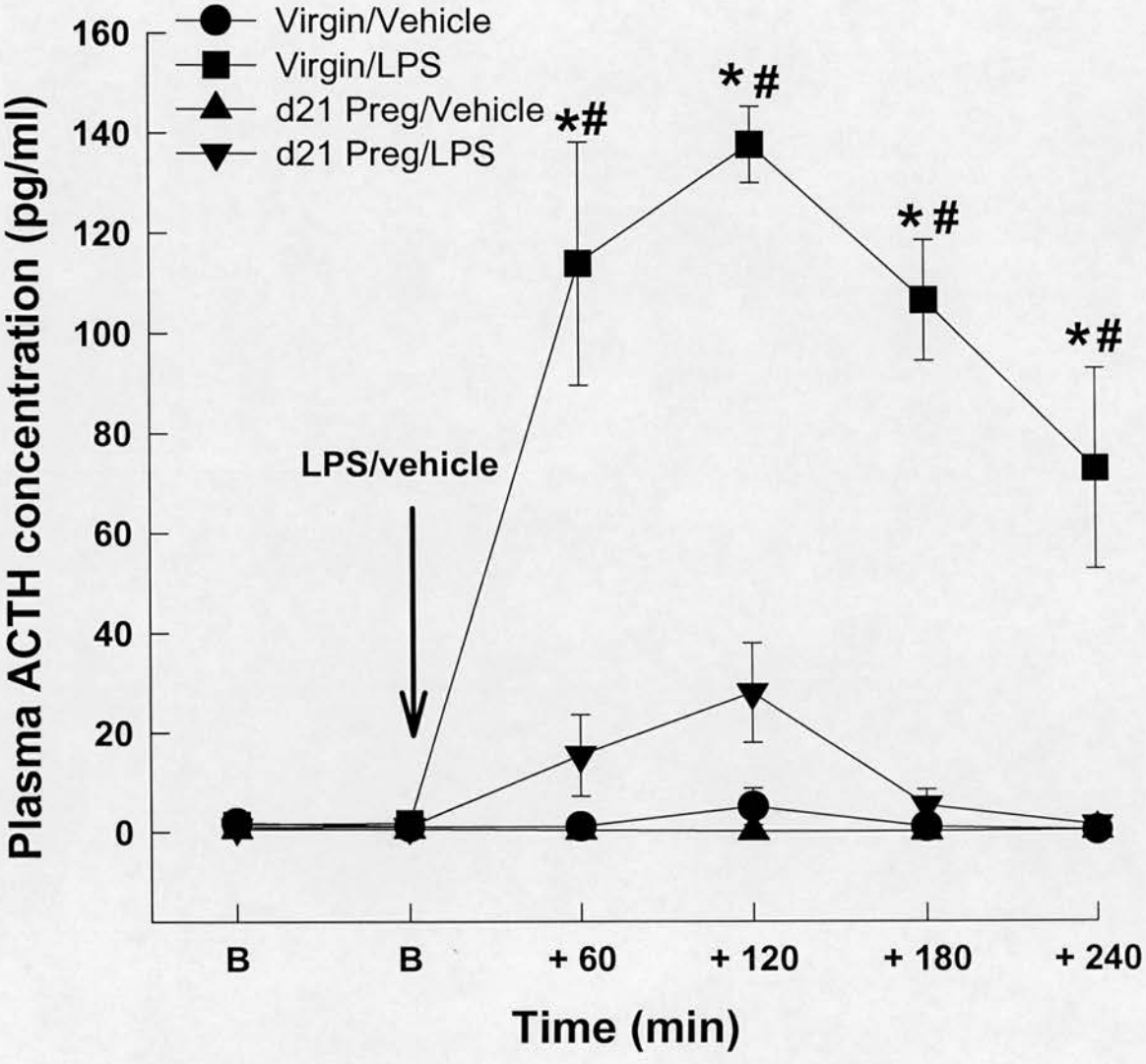


Figure 4.2. The effect of i.v. lipopolysaccharide on plasma ACTH concentrations in virgin and pregnant rats.

Two basal blood samples were collected 30 minutes apart, prior to i.v. administration of either 0.9% saline or 1 μg/kg LPS. Further blood samples were withdrawn 60, 120, 180 and 240 minutes post-injection. Values are plotted as group means ± SEM. Group numbers: virgin/vehicle, n = 10; pregnant/vehicle, n = 7; virgin/LPS, n = 11; pregnant/LPS, n = 10. Two-way ANOVA for repeated measures followed by Student Newman-Keuls multiple comparison test was used for statistical analysis of the data: *$p < 0.001$ vs basal values in the same group; #$p < 0.001$ vs all other groups at the same time point.

Experiment 2

4.3.2. Effects of i.v. IL-1β on plasma ACTH concentration

Basal plasma ACTH concentrations were not significantly different between any of the groups (figure 4.3.). Administration of vehicle (0.5 ml/kg) did not affect plasma ACTH concentrations in either the virgin or the pregnant group. Intravenous administration of IL-1β caused a rapid increase (3.3-fold) in ACTH secretion within 15 min in the virgin group ($p<0.001$; 2-way RM ANOVA; figure 4.3.).
Plasma ACTH concentration remained significantly higher than basal levels for 120 min after IL-1β in the virgin group, and although still elevated at 240 min, levels were not significantly different compared with basal levels. Whereas in the pregnant group, IL-1β induced only a small increase (1.7-fold) in plasma ACTH concentration, 30 min after the injection, however this increase was not significant (figure 4.3.).

4.3.3. Effects of i.v. IL-1β on plasma corticosterone concentration

Basal plasma corticosterone was slightly higher in the pregnant groups, however this was not significant (figure 4.4.). Vehicle administration had no significant effect on corticosterone secretion in either the virgin or the pregnant group. Following IL-1β administration, plasma corticosterone concentration increased significantly within 30 min in the virgin group and was still significantly higher than pre-injection levels at 240 min ($p< 0.001$; 2-way RM ANOVA). Consistent with the ACTH data, i.v. IL-1β induced only a small increase in plasma corticosterone concentration in the pregnant group (figure 4.4.).

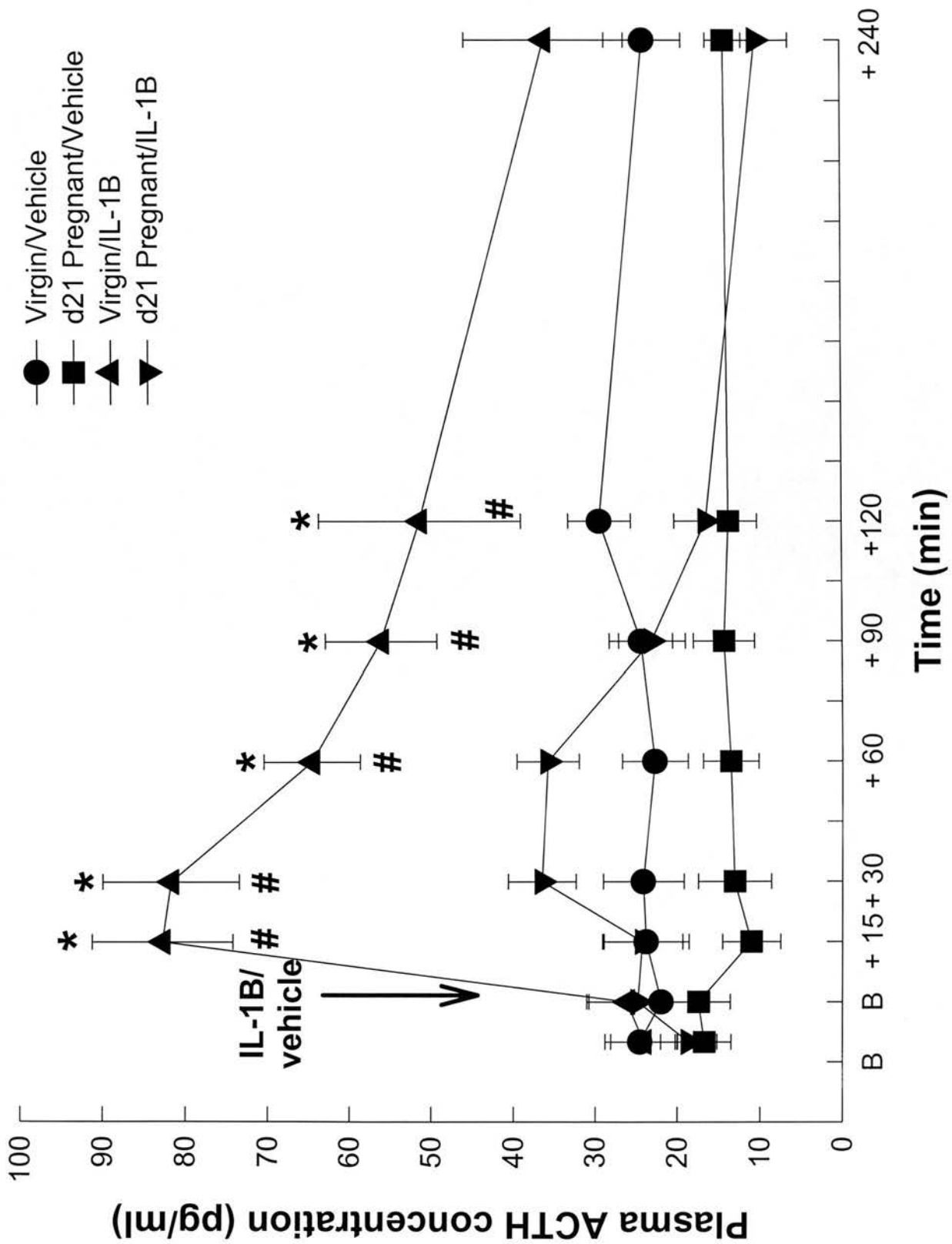

Virgin/Vehicle
d21 Pregnant/Vehicle
Virgin/IL-1B
d21 Pregnant/IL-1B
IL-1B/ vehicle
Plasma ACTH concentration (pg/ml)
Time (min)
B
B + 15
+ 30
+ 60
+ 90
+120
+ 240
0
10
20
30
40
50
60
70
80
90
100

Figure 4.3. The effect of i.v. Interleukin-1β on plasma ACTH concentrations in virgin and pregnant rats.

Two basal blood samples were collected 30 minutes apart, prior to i.v. administration of either 0.2% BSA in PBS or 500 ng/kg rh IL-1β. Further blood samples were withdrawn 15, 30, 60, 90 and 120 minutes post-injection. Rats were killed by decapitation 240 min post-injection and trunk blood was collected for hormone assay. Values are plotted as group means ± SEM. Group numbers: virgin/vehicle, n = 9; pregnant/vehicle, n = 6; virgin/IL-1β, n = 8; pregnant/IL-1β, n = 7. Two-way ANOVA for repeated measures followed by Student Newman-Keuls multiple comparison test was used for statistical analysis of the data: *$p < 0.001$ vs basal values in the same group; #$p < 0.005$ vs all other groups at the same time point.

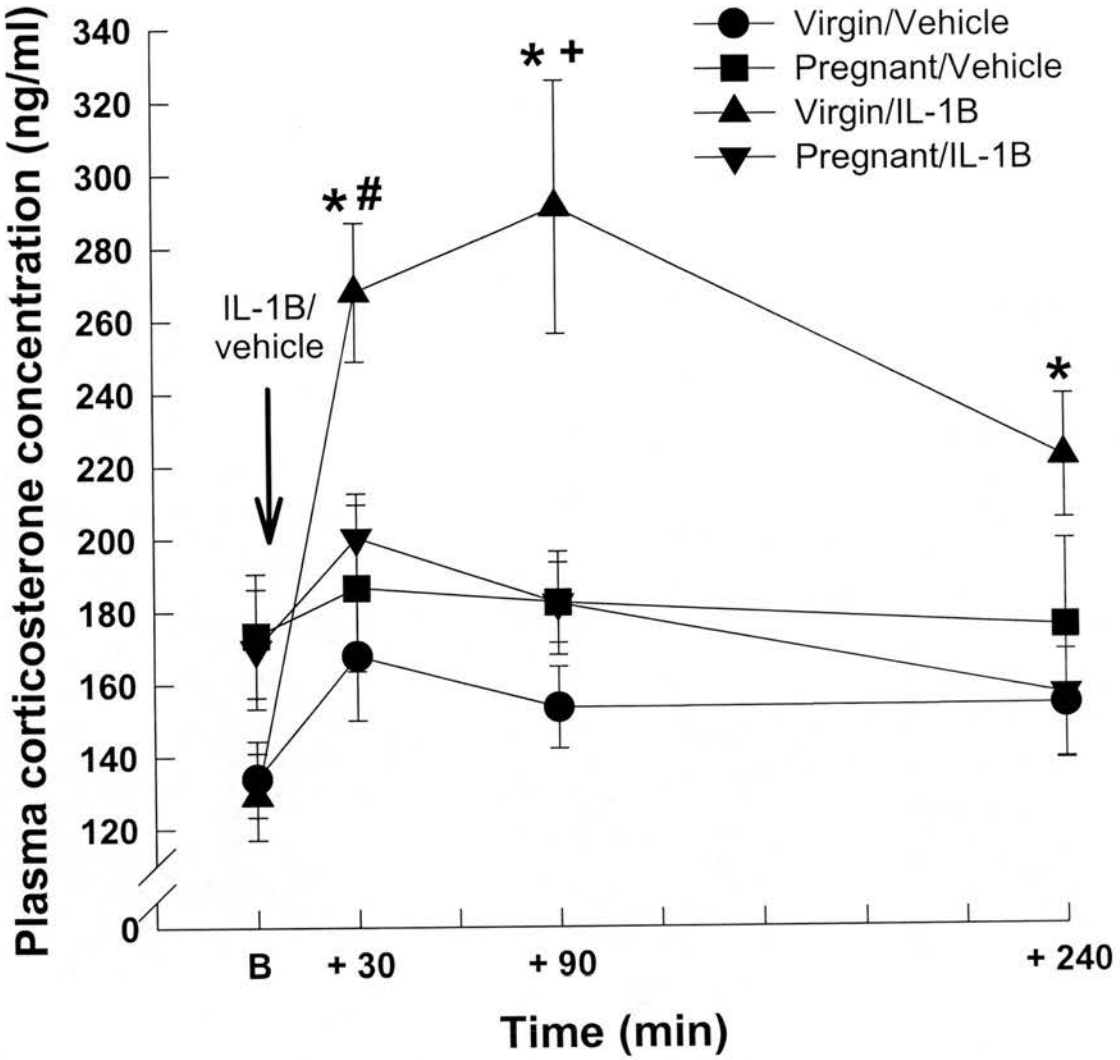


Figure 4.4. The effect of i.v. interleukin-1β on plasma corticosterone concentrations in virgin and pregnant rats.

One basal blood sample was collected prior to i.v. administration of either 0.2% BSA in PBS or 500 ng/kg rh IL-1β. Plasma from blood samples withdrawn 30 and 90 minutes post-injection and from trunk blood collected 240 min post-injection were assayed for corticosterone. Values are plotted as group means ± SEM. Group numbers: virgin/vehicle, n = 9; pregnant/vehicle, n = 6; virgin/IL-1β, n = 8; pregnant/IL-1β, n = 7. Two-way ANOVA for repeated measures followed by Student Newman-Keuls multiple comparison test was used for statistical analysis of the data: *$p < 0.001$ vs basal values in the same group; +$p < 0.001$ and #$p < 0.01$ vs all other groups at the same time point.

4.3.4. Effects of i.v. IL-1β on plasma oxytocin concentration

Plasma oxytocin concentration was not significantly different under basal conditions between any of the groups (figure 4.5.). Vehicle administration had no effect on plasma oxytocin in any of the groups. Following IL-1β administration, plasma oxytocin concentration was significantly increased in the virgin group, however not in the pregnant group, although there was a slight increase (34.1 ± 6.3 pg/ml in the virgin/IL-1β group vs 14.5 ± 8.1 pg/ml in the pregnant group, 15 min after the injection; figure 4.5.).

4.3.5. Effects of i.v. IL-1β on CRH mRNA expression in the pPVN

In the control groups, CRH mRNA expression in the PVN tended to be less in the pregnant animals than in the virgins, though this was not significant (figure 4.5.(a,b)). Four hours after IL-1β administration, CRH mRNA expression (measured by grain area) in the parvocellular region of the PVN was significantly increased in the virgin group ($p<0.001$; 2-way ANOVA), however no change in CRH mRNA expression was observed in the pregnant group (figure 4.6.(a)). Similarly the number of cells within the pPVN expressing CRH mRNA significantly increased following IL-1β administration only in the virgin group (figure 4.6.(b, c)).

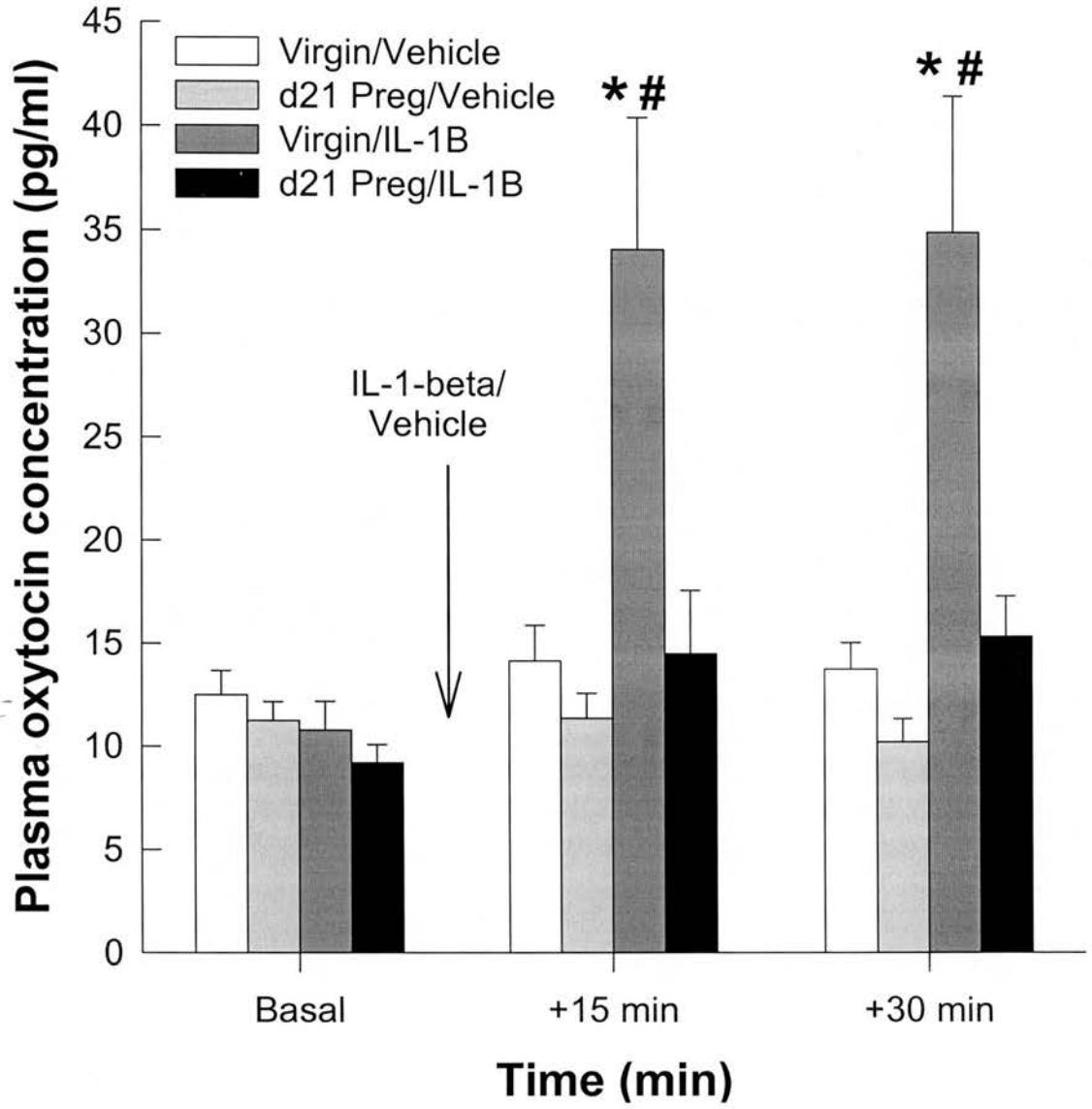


Figure 4.5. The effect of i.v. interleukin-1β on plasma oxytocin concentrations in virgin and pregnant rats.

One basal blood sample was collected prior to i.v. administration of either 0.2% BSA in PBS or 500 ng/kg rh IL-1β. Plasma from blood samples withdrawn 15 and 30 minutes post-injection was assayed for oxytocin. Values are plotted as group means ± SEM. Group numbers: virgin/vehicle, n = 9; pregnant/vehicle, n = 6; virgin/IL-1β, n = 6; pregnant/IL-1β, n = 6. Two-way ANOVA for repeated measures followed by Student Newman-Keuls multiple comparison test was used for statistical analysis of the data: *$p < 0.001$ vs basal values in the same group; #$p < 0.001$ vs all other groups at the same time point.

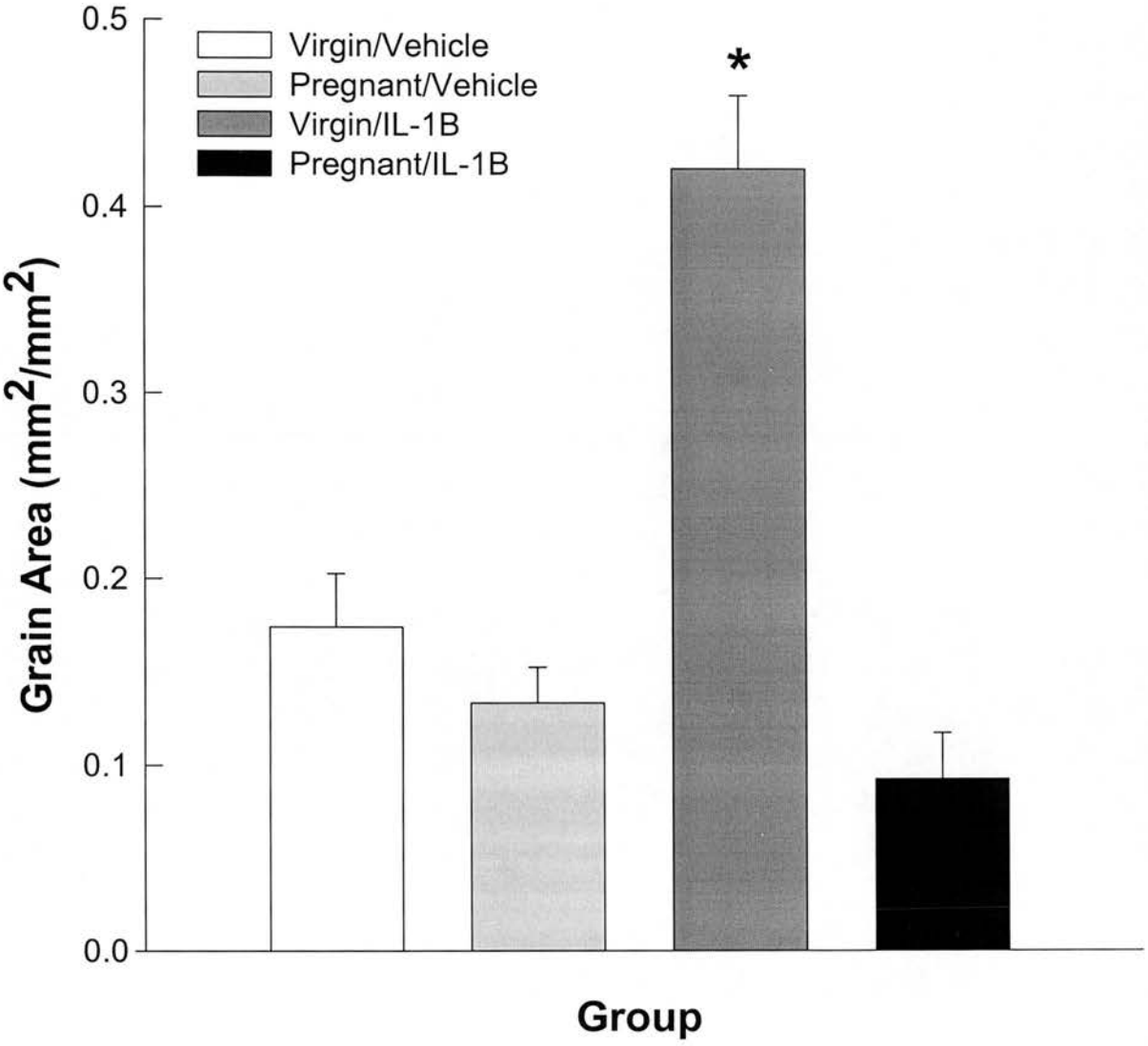


Figure 4.6.(a) The effect of i.v. interleukin-1β on CRH mRNA expression in the parvocellular region of the PVN in virgin and pregnant rats.

Rats were treated i.v. with either 0.2% BSA in PBS or 500 ng/kg rh IL-1β. Four hours after the injection rats were killed by decapitation. Measurements of grain area were made from film autoradiographs over the pPVN. Values are plotted as group means ± SEM. Group numbers: virgin/vehicle, n = 10; pregnant/vehicle, n = 11; virgin/IL-1β, n = 8; pregnant/IL-1β, n = 7. Two-way ANOVA followed by Student Newman-Keuls multiple comparison test was used for statistical analysis of the data: *$p < 0.001$ vs all other groups.

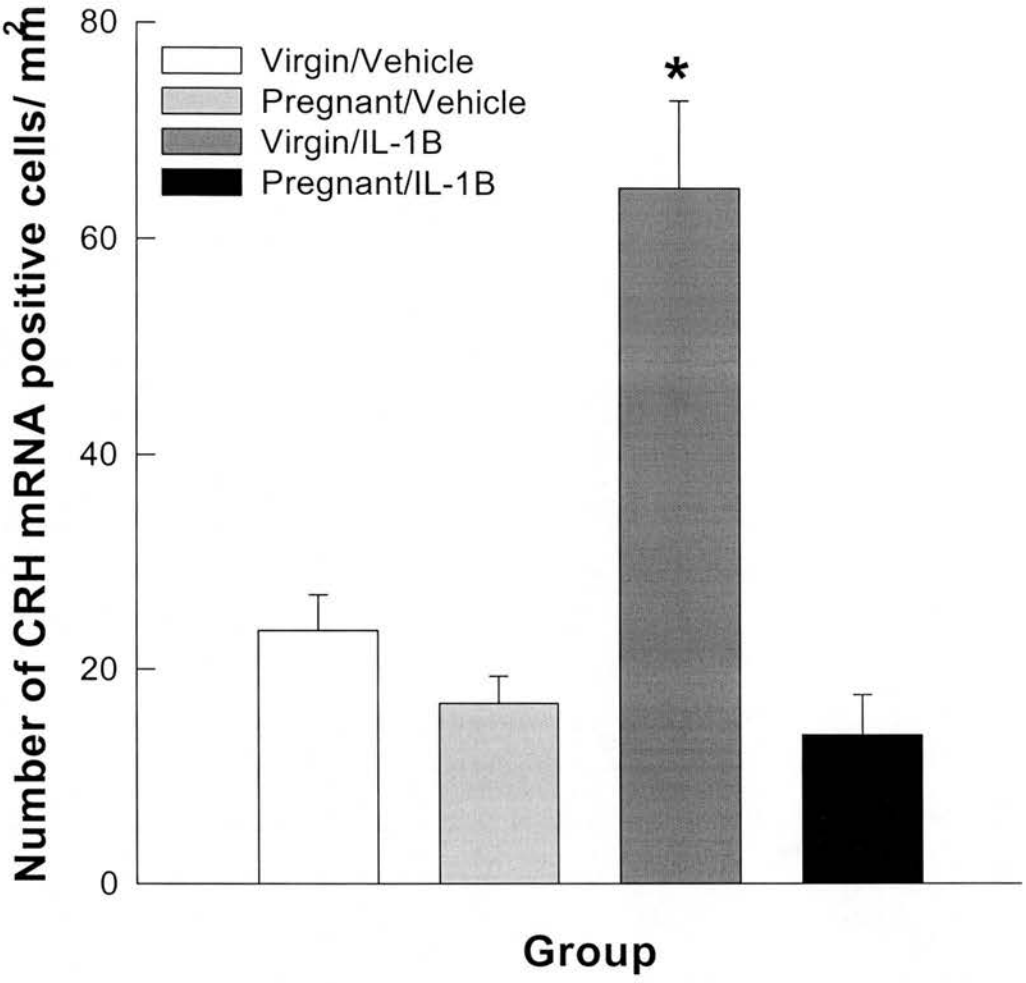


Figure 4.6.(b) The effect of i.v. interleukin-1β on the number of cells expressing CRH mRNA in the parvocellular region of the PVN in virgin and pregnant rats. Rats were treated i.v. with either 0.2% BSA in PBS or 500 ng/kg rh IL-1β. Four hours after the injection rats were killed by decapitation. Cells positive for CRH mRNA expression (defined as a cell with more overlying silver grains than the mean background + 3 standard deviations) were counted in the PVN and the area of the PVN was measured (using NIH Image 1.62 computer software). The data were converted to number of CRH mRNA positive cells per mm^2 and values are plotted as group means ± SEM. Group numbers: virgin/vehicle, n = 10; pregnant/vehicle, n = 11; virgin/IL-1β, n = 8; pregnant/IL-1β, n = 7. Two-way ANOVA followed by Student Newman-Keuls multiple comparison test was used for statistical analysis of the data: *$p < 0.001$ vs all other groups.

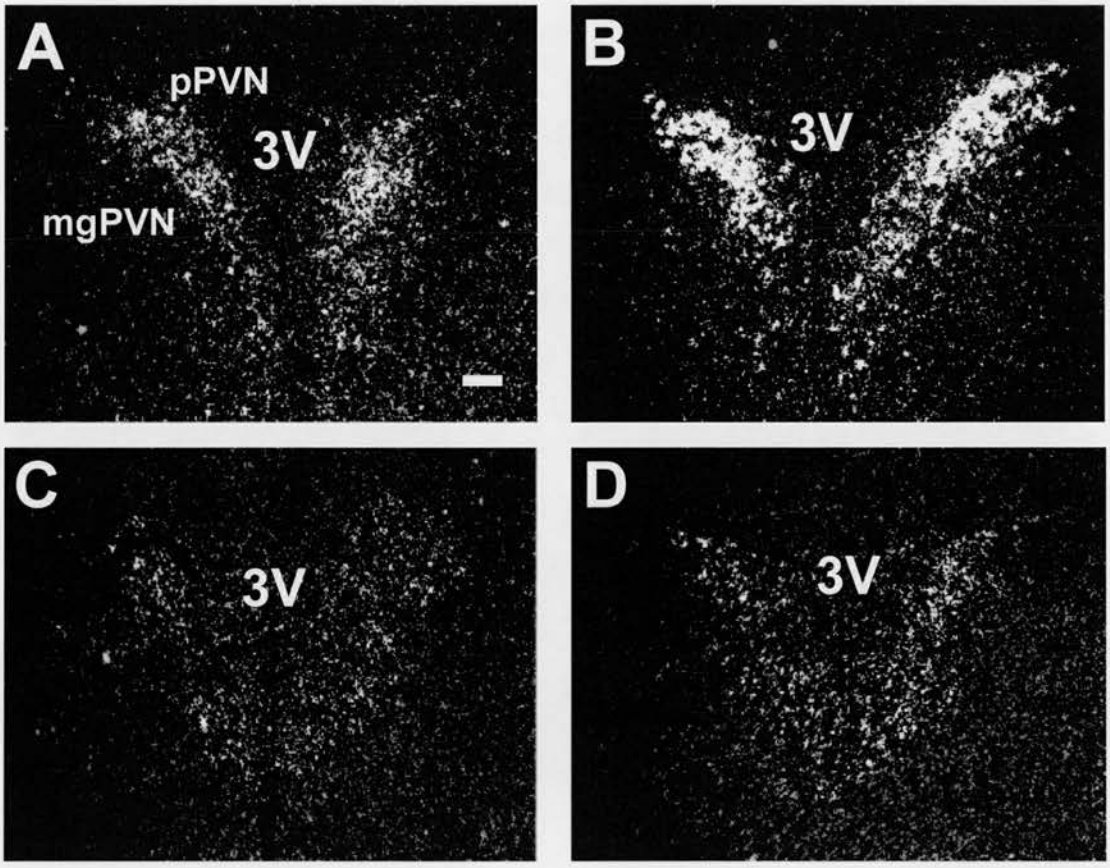


Figure 4.6.(c) The effect of i.v. interleukin-1β on CRH mRNA expression in the parvocellular region of the PVN in virgin and pregnant rats: Photomicrographs. Darkfield autoradiographs of coronal sections through the paraventricular nucleus hybridised with a ^{35}S-labelled oligonucleotide probe complementary to CRH mRNA from: A, virgin/vehicle; B, vigin/IL-1β; C, pregnant/vehicle; D, pregnant/IL-1β. 3V, third ventricle; mgPVN, magnocellular division; pPVN, parvocellular division of PVN. Scale bar: 100μm.

4.3.6. Effects of i.v. IL-1β on Fos expression in the A2 cell region of the NTS

In the vehicle-treated groups, the number of cells expressing Fos in the A2 cell region of the NTS was similar in virgin and pregnant rats. IL-1β administration induced a significant increase in Fos expression in the NTS of both virgin and pregnant rats killed 90 min after the injection (figure 4.7.(a, c)). There was no significant difference in the level of Fos expression in the NTS between the virgin (188.2 ± 25.5 Fos positive nuclei/mm^2) and pregnant (176.7 ± 28.7 Fos positive nuclei/mm^2) IL-1β-treated groups. Similarly, rats killed 4h after IL-1β administration demonstrated increased levels of Fos expression in the NTS, which was not different between virgin and pregnant rats (figure 4.7. (b)).

Experiment 3

4.3.7. Effects of pretreatment with naloxone on plasma ACTH responses to i.v. administration of IL-1β

Basal levels of plasma ACTH were not significantly different between any of the groups. Treatment with either 0.9 % saline or naloxone did not significantly affect basal plasma ACTH concentration in any of the groups (figure 4.8.). IL-1β administration evoked a rapid increase in plasma ACTH concentration in the vehicle treated virgin group which peaked within 15 min (63.5 ± 2.8 pg/ml) and this response was significantly attenuated in the vehicle treated pregnant group (17.9 ± 3.5 pg/ml). Pretreatment with naloxone had no significant effect on the ACTH secretory response to systemic IL-1β in the virgin group (figure 4.8.), however in the pregnant group, naloxone significantly enhanced ACTH secretion in response to IL-1β ($p < 0.001$; two-way RM ANOVA). Plasma ACTH concentration in the naloxone treated pregnant group did not reach the levels observed in the virgin groups following IL-1β administration (figure 4.8.).

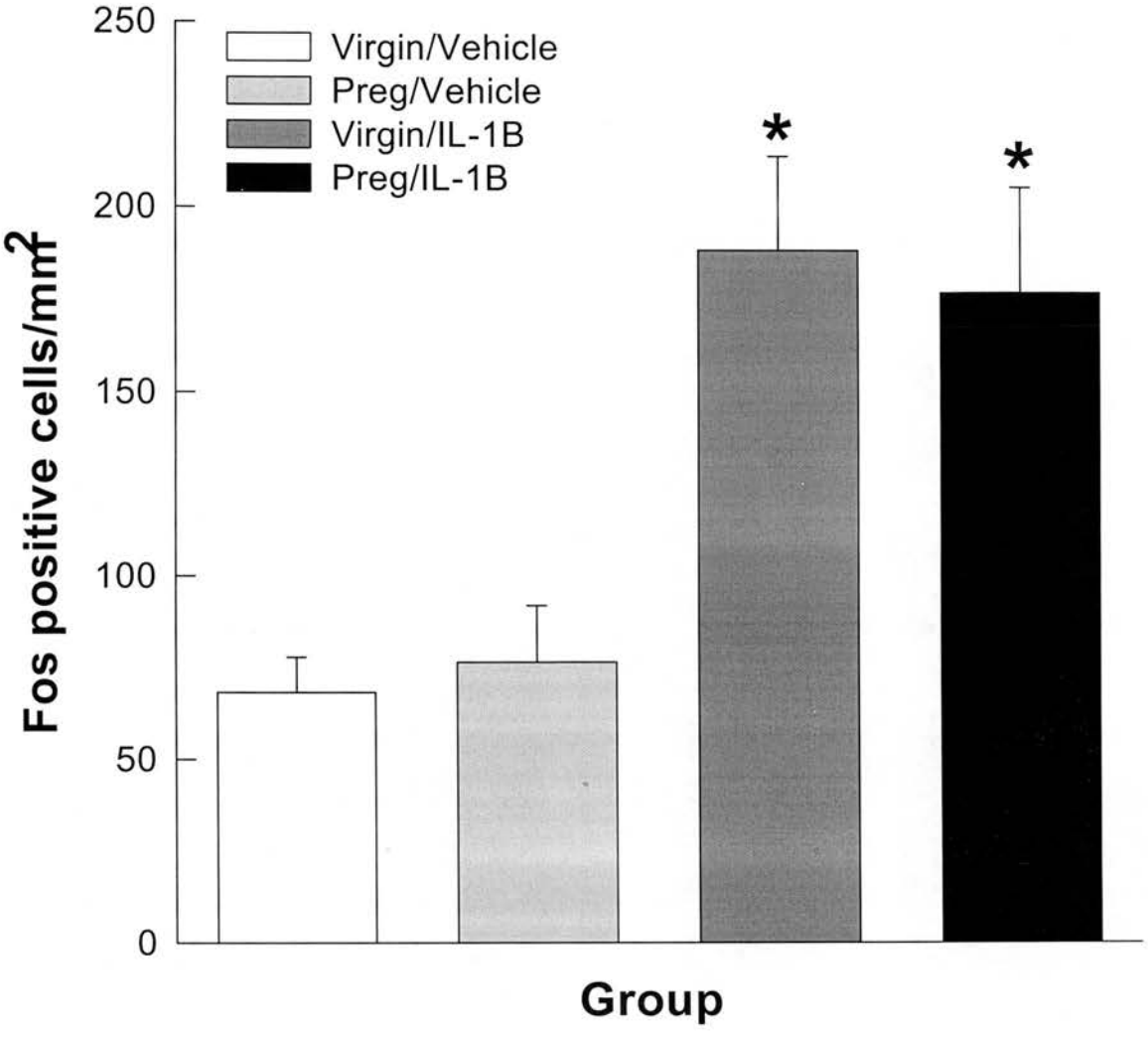


Figure 4.7.(a) The effect of i.v. interleukin-1β on Fos expression (at 90 min) in the A2 cell region of the NTS in virgin and pregnant rats.

Rats were treated i.v. with either 0.2% BSA in PBS or 500 ng/kg rh IL-1β. 90 min after the injection rats were killed by decapitation. Brainstem sections were cut on a cryostat and processed for Fos immunocytochemistry. Fos positive cells were counted in the A2 cell region of the NTS (at the level of the area postrema, ~ 13.5-14.0 mm caudal to Bregma) and the area of the NTS was measured using NIH Image 1.62 software. The data were then converted to Fos positive cells per mm^2. Values are plotted as group means ± SEM. Group numbers: virgin/vehicle, n = 6; pregnant/vehicle, n = 7; virgin/IL-1β, n = 6; pregnant/IL-1β, n = 8. Two-way ANOVA followed by Student Newman-Keuls multiple comparison test was used for statistical analysis of the data: *$p < 0.001$ vs vehicle.

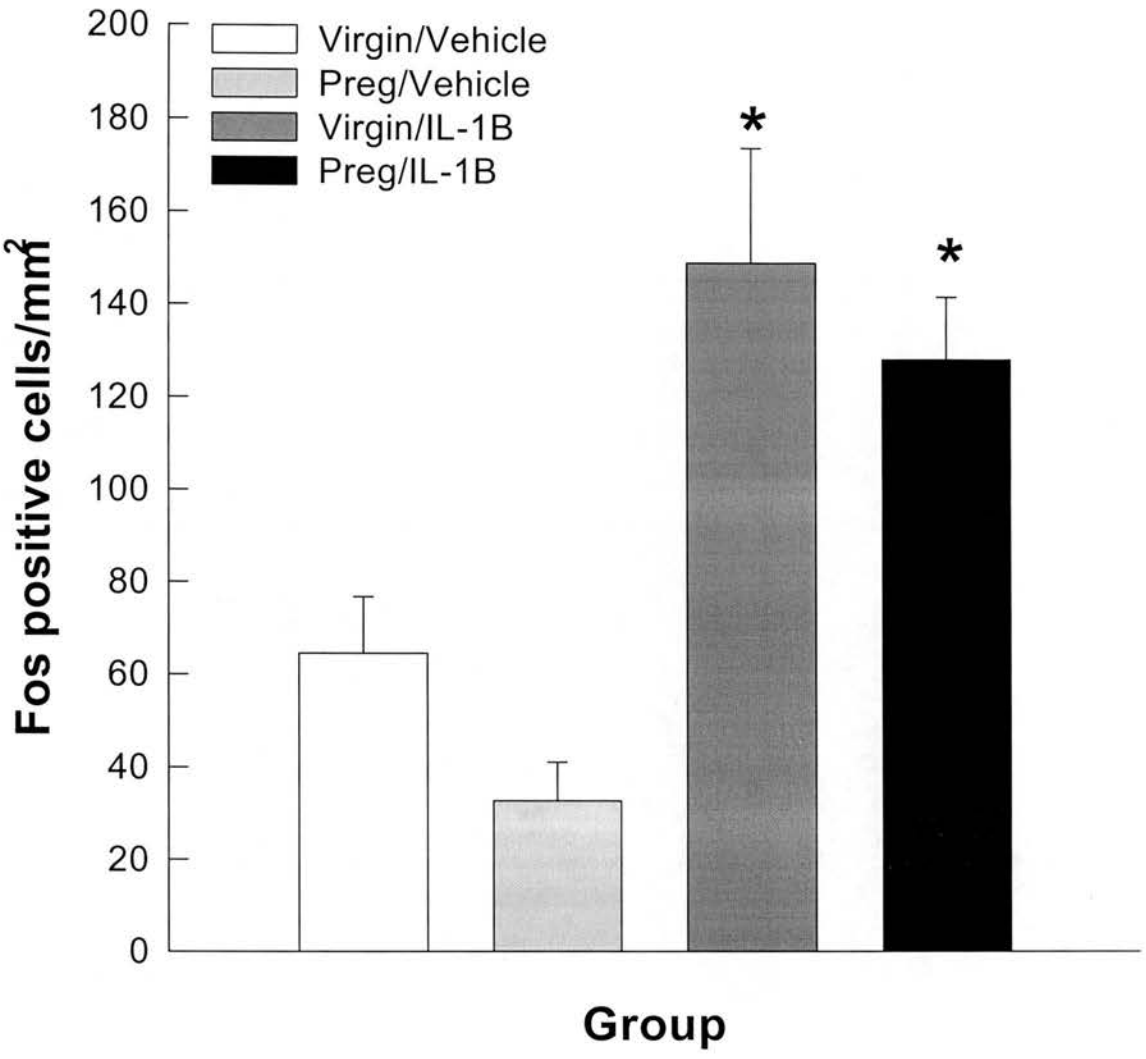


Figure 4.7.(b) The effect of i.v. interleukin-1β on Fos expression (at 4h) in the A2 cell region of the NTS in virgin and pregnant rats.

Rats were treated i.v. with either 0.2% BSA in PBS or 500 ng/kg rh IL-1β. Four hours after the injection rats were killed by decapitation. Brainstem sections were cut on a cryostat and processed for Fos immunocytochemistry. Fos positive cells were counted and data is expressed as before (see figure 4.7.(a) legend). Values are plotted as group means ± SEM. Group numbers: virgin/vehicle, n = 10; pregnant/vehicle, n = 11; virgin/IL-1β, n = 7; pregnant/IL-1β, n = 7. Two-way ANOVA followed by Student Newman-Keuls multiple comparison test was used for statistical analysis of the data: *$p < 0.001$ vs vehicle.

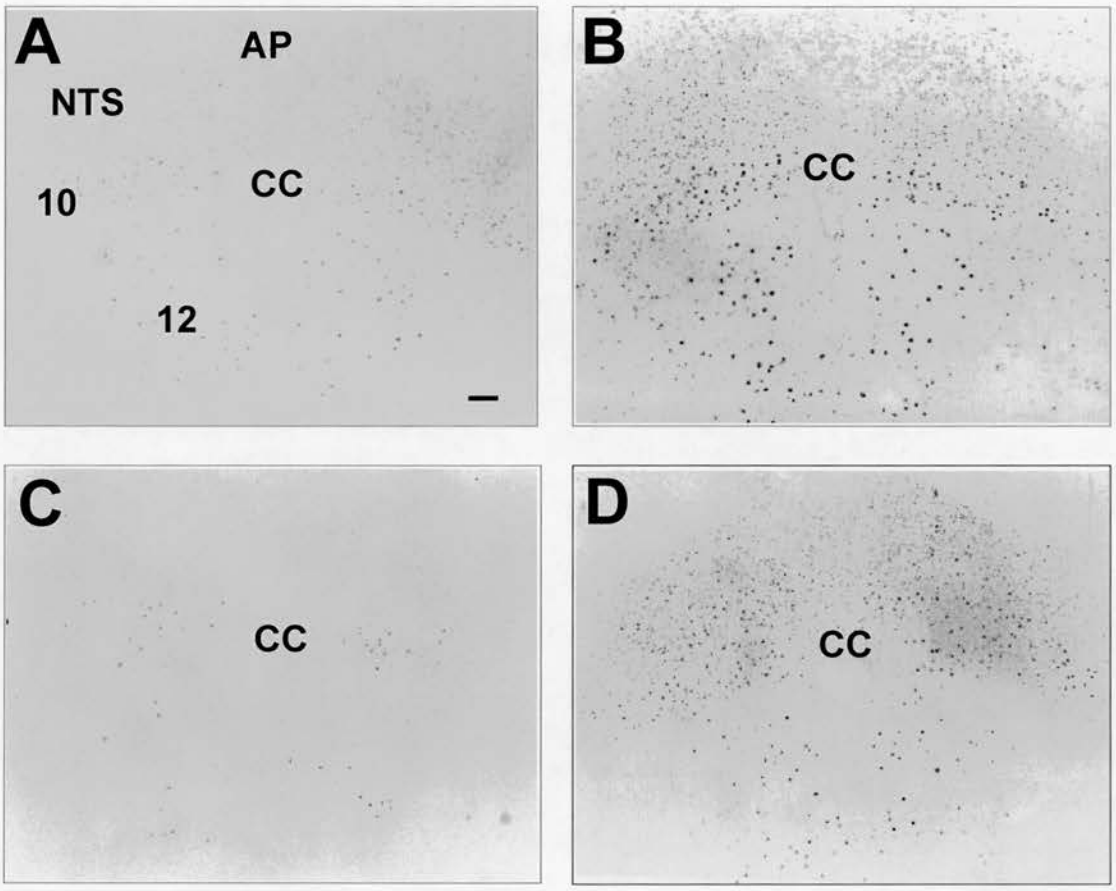


Figure 4.7.(c) The effect of i.v. interleukin-1β on Fos expression (at 90 min) in the A2 cell region of the NTS in virgin and pregnant rats: Photograph.

Coronal sections through rat brainstem showing Fos immunoreactivity in the nucleus of the tractus solitarius (NTS) from: A, virgin/vehicle; B, virgin/IL-1β; C, pregnant/vehicle; D, pregnant/IL-1β. AP, area postrema, CC, central canal; 10, dorsal motor nucleus of vagus; 12, hypoglossal nucleus. Scale bar = 100μm.

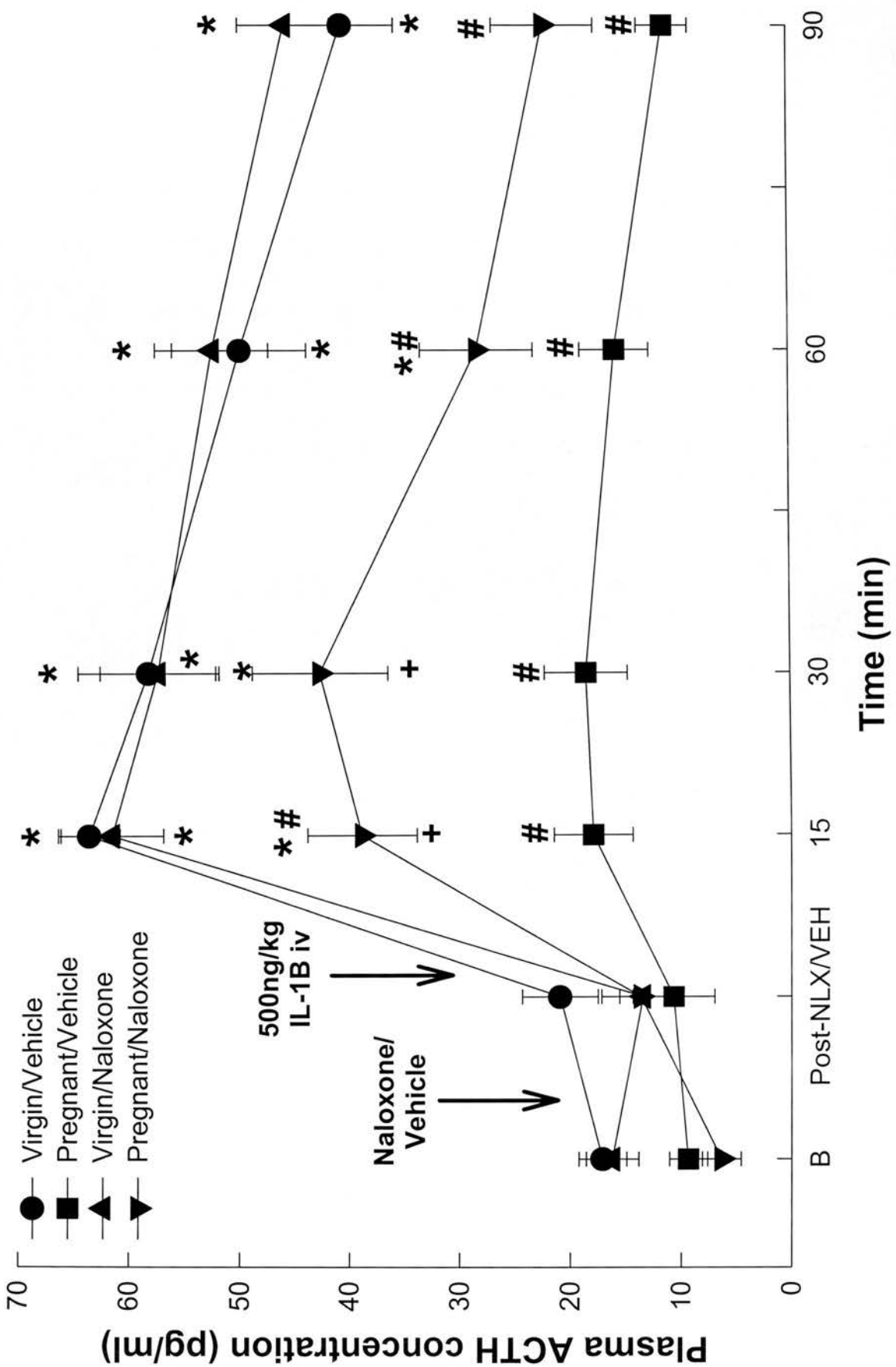

Plasma ACTH concentration (pg/ml)
0
10
20
30
40
50
60
70
B
Post-NLX/VEH
15
30
60
90
Time (min)
Virgin/Vehicle
Pregnant/Vehicle
Virgin/Naloxone
Pregnant/Naloxone
500ng/kg
IL-1B iv
Naloxone/
Vehicle

Figure 4.8. The effect of pretreatment with naloxone on plasma ACTH responses to i.v. interleukin-1β in virgin and pregnant rats.

One basal blood sample was collected, prior to i.v. administration of either 0.9% saline or 5 mg/kg naloxone (10 mg/ml). A blood sample was withdrawn 15 min after the naloxone/vehicle injection, which was immediately followed by i.v. administration of 500 ng/kg rh IL-1β (1μg/ml) given to all rats. Sequential blood samples were taken 15, 30, 60 and 90 min after IL-1β administration. Values are plotted as group means ± SEM. Group numbers: virgin/vehicle, n = 7; pregnant/vehicle, n = 6; virgin/naloxone, n = 8; pregnant/naloxone, n = 6. Two-way ANOVA for repeated measures followed by Student Newman-Keuls multiple comparison test was used for statistical analysis of the data: *$p < 0.001$ vs basal values in the same group; #$p < 0.001$ vs virgin/vehicle group; +$p < 0.02$ vs all other groups at the same time point.

Experiment 4

4.3.8. Effects of pretreatment with naloxone on plasma oxytocin responses to i.v. administration of IL-1β

Basal plasma oxytocin concentration did not differ between any of the groups. Vehicle administration had no effect on oxytocin secretion in either the virgin or the pregnant groups (figure 4.9.). Naloxone treatment induced a small increase in plasma oxytocin concentration in both groups, however this increase was not significant. As before (see section 4.3.4.) administration of IL-1β evoked a significant increase (5.4-fold) in oxytocin secretion within 15 min of the injection in the virgin vehicle treated group but had no effect in the pregnant vehicle treated group (figure 4.9.). Naloxone pretreatment seemed to further enhance the oxytocin secretory response to IL-1β in virgin rats, although this was not significant, whereas in the pregnant group, naloxone significantly increased (8.4-fold and 12.4-fold increase at 15 and 30 min post-IL-1β injection, respectively) the oxytocin secretory response to IL-1β, which was greater than in both virgin groups (figure 4.9.).

4.3.9. Effects of naloxone pretreatment on CRH gene responses in the pPVN to i.v. IL-1β

CRH mRNA expression in the pPVN 4 hours after IL-1β administration was 7.4-fold greater in the virgin vehicle treated rats compared with the pregnant vehicle treated rats (figure 4.10.(a)). Naloxone treatment significantly increased CRH mRNA expression in response to IL-1β in the pregnant group (447% of vehicle treated pregnant group) and significantly reduced CRH mRNA expression in response to IL-1β in the virgin group (66% of vehicle treated virgin group).

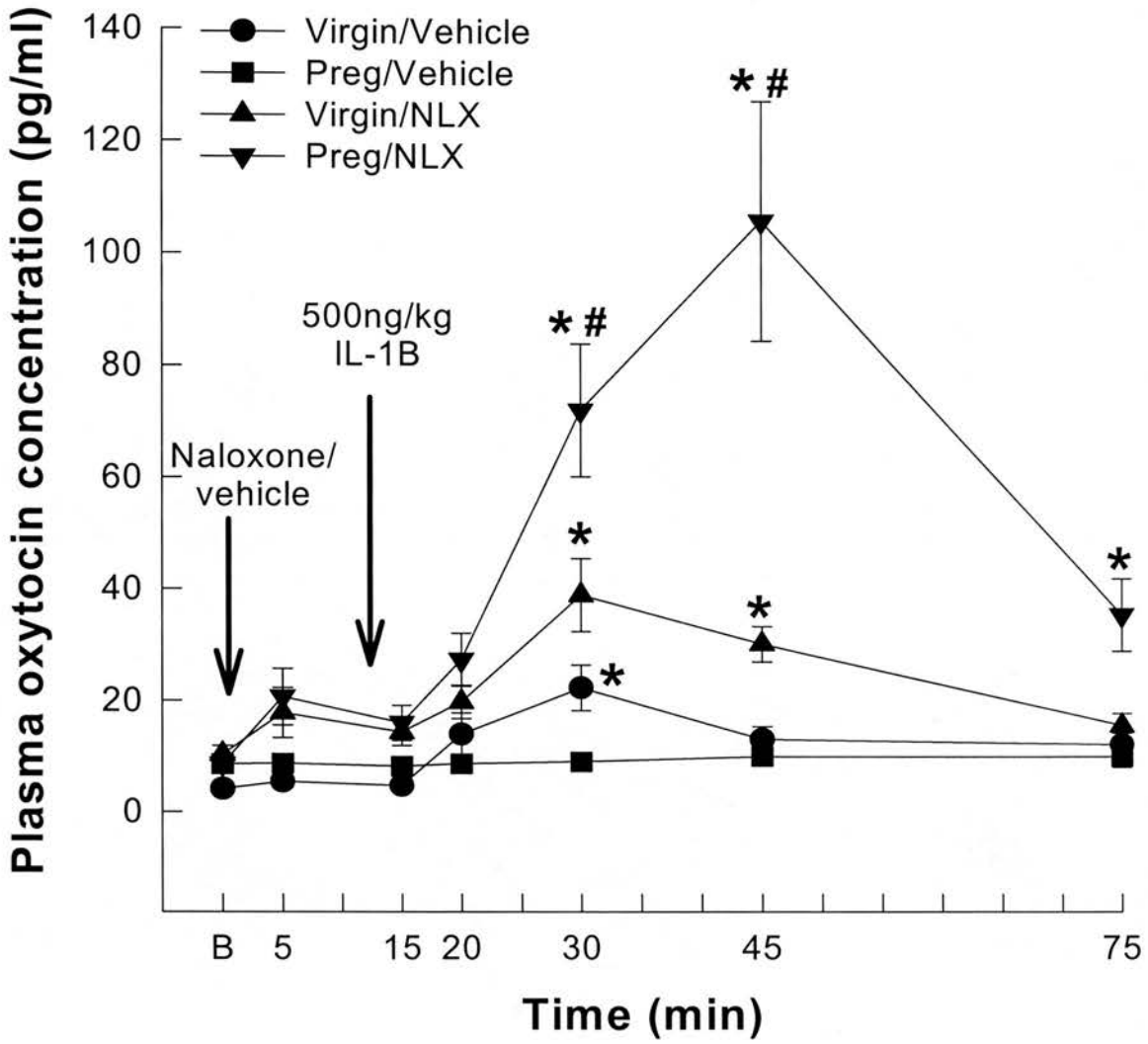


Figure 4.9. The effect of pretreatment with naloxone on plasma oxytocin responses to i.v. interleukin-1β in virgin and pregnant rats.

One basal blood sample was collected, prior to i.v. administration of either 0.9% saline or 5 mg/kg naloxone (10 mg/ml). Further blood samples were withdrawn 5 and 15 min after the naloxone/vehicle injection, which was immediately followed by i.v. administration of 500 ng/kg rh IL-1β (1μg/ml). Sequential blood samples were taken 5, 15, 30 and 60 min after IL-1β administration. Values are plotted as group means ± SEM. Group numbers: virgin/vehicle, n = 6; pregnant/vehicle, n = 6; virgin/naloxone, n = 6; pregnant/naloxone, n = 6. Two-way ANOVA for repeated measures followed by Student Newman-Keuls multiple comparison test was used for statistical analysis of the data: *$p < 0.05$ vs basal values in the same group; #$p < 0.05$ vs all other groups at the same time point.

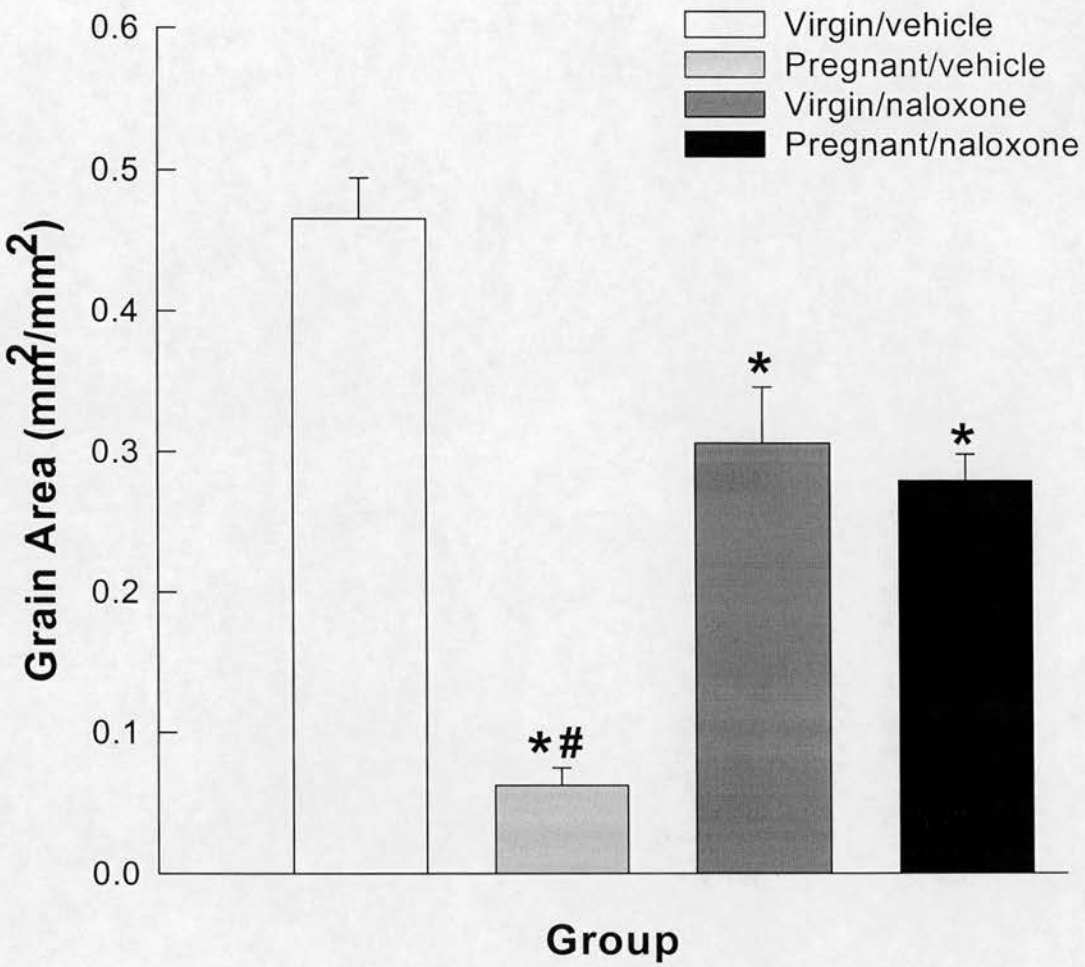


Figure 4.10.(a) The effect of naloxone pretreatment on CRH mRNA expression in the parvocellular region of the PVN following i.v. interleukin-1β in virgin and pregnant rats.

Rats were treated with either 0.9% saline or 5 mg/kg naloxone (10 mg/ml) i.v. followed 15 min later by an i.v. injection of 500 ng/kg rh IL-1β (1μg/ml) in all rats. Four hours after the IL-1β injection the rats were killed by decapitation. Measurements of grain area were made from film autoradiographs over the pPVN using the NIH Image 1.62 software package. Values are plotted as group means ± SEM. Group numbers: virgin/vehicle, n = 6; pregnant/vehicle, n = 6; virgin/naloxone, n = 6; pregnant/naloxone, n = 7. Two-way ANOVA followed by Student Newman-Keuls multiple comparison test was used for statistical analysis of the data: *$p < 0.001$ vs virgin/vehicle group; #$p < 0.001$ vs all other groups.

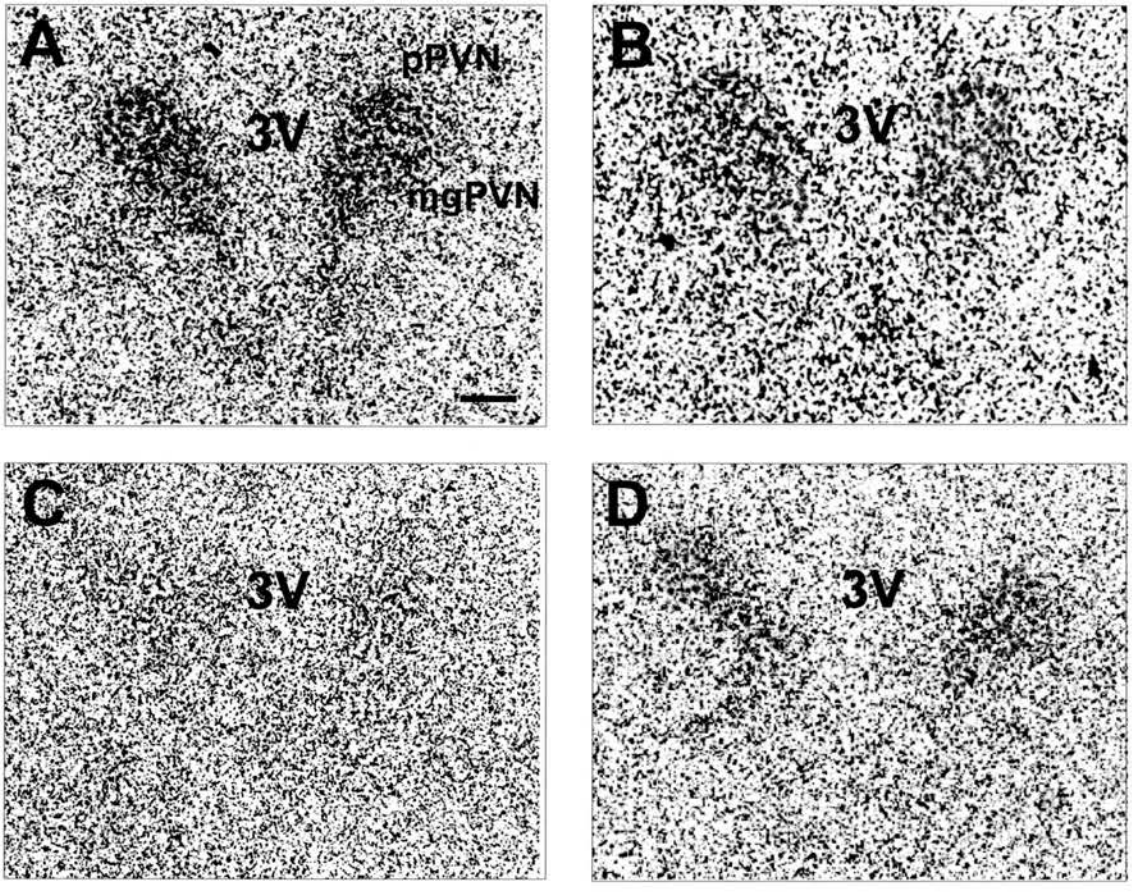


Figure 4.10.(b) The effect of naloxone pretreatment on CRH mRNA expression in the parvocellular region of the PVN following i.v. interleukin-1β in virgin and pregnant rats: Photomicrographs.

Brightfield photographs of autoradiographic film exposed for 21 days to coronal brain sections hybridised with a ^{35}S-labelled oligo-probe complementary to CRH mRNA from: A, virgin/vehicle; B, virgin/naloxone; C, pregnant/vehicle; D, pregnant/naloxone. 3V, third ventricle; mgPVN, magnocellular division of PVN; pPVN, parvocellular division of PVN. Scale bar = 100μm.

4.4. Discussion

The aim of the present set of experiments was to test whether the responsiveness of the HPA axis to a specific physical stressor is attenuated in pregnancy as it is to emotional stressors. The data clearly demonstrate that the HPA axis is less responsive to systemic immune signals in pregnancy, consistent with the results observed following exposure to emotional stressors in pregnancy (see chapter 3).

Activation of the HPA axis following immune challenge

The results from experiment 1 demonstrate that the HPA axis is activated (reflected by ACTH secretion) following immune challenge with i.v. LPS in non-pregnant female rats. These data are consistent with previous studies in male rats (Takemura *et al* , 1997; Turnbull *et al* , 1998). However during late pregnancy (day 21) the ACTH secretory response to peripheral administration of LPS was strongly and significantly attenuated. This attenuated HPA axis response to endotoxin induced immune challenge does not occur as a result of changes in cytokine generation in pregnancy, since the ACTH and corticosterone secretory responses were also markedly reduced following stimulation with the cytokine, IL-1β (experiment 2). These changes in pregnancy are comparable to the attenuation of HPA axis responses previously discussed following exposure to emotional stressors (restraint and maternal defence) in chapter 3.

These results are unlikely to reflect an enhanced metabolic clearance of corticosterone during pregnancy, since the clearance rate of corticosterone is reported not to differ between virgin and pregnant rats (Waddell & Atkinson, 1994). It is more likely that the attenuated pituitary and adrenal gland responses to immune challenge are a consequence of reduced CRH drive. Indeed, CRH mRNA expression in the pPVN was not stimulated by i.v. IL-1β administration in the pregnant group as it was in the virgin rats. If it is assumed that CRH gene activation (reflected by increased CRH mRNA expression) reflects stimulation of the CRH cell bodies which

simultaneously causes release of pre-formed CRH from the nerve terminals, it is likely that less CRH is released from the nerve terminals at the median eminence in response to IL-1β in the pregnant rats, thus explaining the attenuated ACTH and corticosterone responses.

Reduced activation of CRH mRNA expression in the pregnant rats following IL-1β could be a consequence of altered neural input. CRH expression is under tonic stimulatory influence from ascending noradrenergic and adrenergic pathways (Kiss *et al*, 1996). Thus if these stimulatory pathways are tonically inhibited or opposed by an inhibitory input (e.g. GABAergic projections) this could explain why the CRH neurones are non-responsive in pregnancy. NAergic projections from the NTS to the PVN have been shown to be important in relaying information from the periphery to the PVN neurones following activation of the immune system. To test whether these inputs were activated less by peripheral administration of IL-1β in pregnant rats, Fos (an immediate-early gene which is rapidly up-regulated when a neurone is activated, therefore it provides a good indicator of neuronal activation) expression was evaluated in the A2 cell region (where NA cells are located) of the NTS in the brainstem. I.v. IL-1β induced similar levels of Fos expression in the A2 cell region of the NTS in the virgin and pregnant groups, indicating that the NTS neurones were not differently activated by IL-1β during pregnancy. These results demonstrate that IL-1β signalling to the noradrenergic NTS neurones is intact in the pregnant rats. Thus it seems unlikely that signalling mechanisms from blood vessels in response to systemic IL-1β (involving generation of prostaglandins via COX pathways) are affected in pregnancy and instead the "breakdown in communication" which prevents the CRH neurones from responding to systemic IL-1β occurs somewhere on a pathway between the NTS neurones and the PVN CRH neurones. Whether the neurones in the NTS that are activated by IL-1β are noradrenergic neurones needs to be verified with immunocytochemical double-labelling techniques. There are several possibilities as to what is happening. When the ascending noradrenergic projections from the brainstem to the hypothalamus are lesioned bilaterally (by intracerebral injection of 6-hydroxydopamine), there is a significant reduction in ACTH secretion

and increases in PVN CRH mRNA expression in response to i.v. IL-1β are virtually abolished (Melik Parsadaniantz *et al*, 1995). Thus, brainstem neurones projecting rostrally, including those projecting to the pPVN, are essential in mediating HPA axis responses to IL-1β. Here activation of the brainstem NTS neurones was similar in the virgin and pregnant rats, however it is not known whether these neurones were noradrenergic, and if so, whether NA release from the nerve terminals was similar in the two groups. Since NA has been shown to have an excitatory effect on CRH neurones, differences in NA release may explain the differences in CRH neurone activation in response to IL-1β. To establish if this is the case it would be necessary to measure NA release in the PVN by microdialysis following administration of IL-1β. It is not clear whether changes in NA release in pregnancy would be mediated through pre- (e.g. inhibition of NA release) or post-synaptic (such as antagonism at NA receptors or inhibition of the signalling pathways activated by NA binding its receptor) actions.

In addition to the direct input pathway from the NTS neurones to the PVN, it has been proposed that brainstem catecholamine cells in the NTS and VLM may also mediate HPA axis responses to systemic IL-1β indirectly via the CeA and the BNST (Buller *et al*, 2001). Therefore if signalling from the NTS to the PVN via these forebrain structures were somehow disrupted in pregnancy, this could also account for the reduced responsiveness of the HPA axis to IL-1β.

Activation of the oxytocin system following immune challenge

The oxytocin secretory response to immune challenge with systemic IL-1β was markedly less in the pregnant group compared with the virgin group. It is likely that hypothalamic oxytocin neurones are activated following systemic IL-1β by a similar brainstem input as the CRH neurones. The A2 cell group in the NTS provides a direct excitatory input to oxytocin neurones in the SON and PVN (Onaka *et al*, 1995) and combined retrograde tracing and immunocytochemical studies have shown that a high proportion of these neuronal inputs are catecholaminergic (Meddle *et al*, 2000). Stimulation of this pathway with intravenous cholecystokinin (CCK; which acts via

the vagus nerve (Smith *et al*, 1981) to activate brainstem NTS neurones (Luckman, 1992)) activates oxytocin cell bodies in the SON (Verbalis *et al*, 1991), resulting in increased firing rate of oxytocin neurones, increased oxytocin secretion and increased Fos expression in the SON (Onaka *et al* , 1995). A large body of evidence indicates that activation of the SON oxytocin neurones by CCK stimulation is mediated by noradrenaline. Indeed, i.v. CCK induces Fos expression in a noradrenergic population of cells in the NTS (Luckman, 1992), increases NA release in the SON and PVN (Kendrick *et al*, 1991) and destruction of the noradrenergic innervation to the SON (by selective neurotoxins) blocks Fos expression in the SON ipsi-lateral to the lesion, but not in the contra-lateral SON or in the NTS (Onaka *et al* , 1995). Furthermore, recent evidence suggests that activation of the oxytocin neurones in the SON at parturition involve a noradrenergic brainstem pathway (Meddle *et al* , 2000; Douglas *et al*, 2001). Thus in the present experiments it is possible that oxytocin secretion in the virgin rats following systemic administration of IL-1β is a consequence of activation of a noradrenergic pathway from the NTS. If this is the case then it is likely that, noradrenergic signalling is interrupted between the NTS and the magnocellular oxytocin neurones in late pregnancy, since the NTS neurones were activated similarly by IL-1β in virgin and pregnant rats.

Effect of opioids on HPA axis responses to immune challenge

Endogenous opioids have previously been shown to actively inhibit oxytocin secretion following stress in late pregnancy (Douglas *et al*, 1993; Douglas *et al*, 1995). More recently it has been shown that blocking the actions of endogenous opioids with naloxone, causes a reduction in the ACTH secretory response to forced swimming in virgin female rats and evokes a moderate (though not significant) increase in ACTH secretion in late pregnant rats exposed to forced swimming (Douglas *et al* , 1998). Thus it seemed plausible that opioids may act to restrain HPA axis responses to IL-1β in pregnancy. Here pretreatment with naloxone did indeed reinstate an ACTH response to IL-1β administration in the pregnant group, although ACTH secretion in the pregnant rats did not reach the levels observed in the virgin group. These data strongly suggest a role for endogenous opioids in the attenuated

ACTH secretory response to peripheral administration of IL-1β. It is likely that some other factor contributes to the attenuated HPA axis responses to IL-1β in pregnancy since naloxone treatment only partially restored an ACTH response in the pregnant rats. However the ACTH secretory response to IL-1β in the pregnant rats may not be maximal due to reduced stimulation of pituitary corticotropes by AVP as a result of reduced drive to the pPVN AVP neurones in late pregnancy (reflected by decreased AVP hnRNA expression, see Chapter 3).

Naloxone pretreatment also restored a response to IL-1β at the level of the CRH neurones in the pregnant group, as indicated by increased CRH mRNA expression. In contrast to the lack of effect of naloxone on the ACTH response in virgin rats, naloxone resulted in an attenuated PVN CRH mRNA response to IL-1β in the virgin group. A similar effect of naloxone has previously been reported on ACTH secretion in virgin rats (Douglas *et al* , 1998). The apparent discrepancy between the ACTH and CRH mRNA results in the virgin-naloxone treated group may reflect that the relationship between CRH neurone activation and the ACTH secretory response was at the top end of the CRH-ACTH dose-response curve. The CRH mRNA data indicate that the effects of endogenous opioids switch from being *excitatory* in non-pregnant rats to being *inhibitory* in pregnant rats. Indeed, pretreatment with morphine has been shown to potentiate stress-induced increases in hypothalamic CRH and plasma ACTH levels in vivo (Buckingham, 1982) in male rats. Furthermore, incubation of isolated hypothalami (from male rats) with either morphine, met-enkephalin or leu-enkephalin induces CRH secretion, effects which can be blocked by treatment with naloxone (Buckingham, 1982). Here, administration of naloxone prior to stimulation with IL-1β has revealed an inhibitory action of opioids on HPA activity in the pregnant rats. A similar effect has been reported following forced swimming (Douglas *et al* , 1998), where blocking the actions of opioids with naloxone resulted in an attenuated ACTH secretory response to forced swimming in virgin rats, but caused a modest increase in ACTH secretion in the pregnant rats exposed to swim stress. Together these data further support a role for differential actions of opioids in virgin and pregnant rats.

The opioid receptor type involved in mediating these effects on HPA activity cannot be established from these experiments, however it is possible that the opposing actions of opioids observed in virgin and pregnant rats is mediated through different opioid receptor subtypes. At the dose used, naloxone will be able to act on all opiate receptors and specific receptor antagonists are necessary to ascertain the receptors involved. Previous studies have indicated that the hypothalamus is almost completely devoid of δ-binding sites (Mansour *et al*, 1987) suggesting that these receptors are unlikely candidates in HPA axis regulation. Blockade of μ-receptors with specific antagonists abolishes the ACTH response to surgical stress in male rats (Cover & Buckingham, 1989) suggesting that opioids acting on these receptors mediate an excitatory effect on HPA activity. Thess data together with the present findings suggest that in the virgin rats naloxone may be blocking μ-receptors, thus causing an attenuated CRH mRNA response to systemic IL-1β administration. In contrast, blockade of κ-receptors results in exaggeration and prolongation of the stress response (Cover & Buckingham, 1989). In the pregnant group naloxone may be acting by blocking opioid binding to κ-receptors, thus removing a tonic inhibitory effect on HPA activity. This could explain the restoration of a HPA response to IL-1β in the pregnant group pretreated with naloxone. On the other hand, pregnancy may involve activation of an inhibitory μ-opioid mechanism that functions to restrain HPA activity, as is the case for endogenous opioid restraint of oxytocin neurones at this time (Douglas *et al* , 1995).

The origin of the endogenous opioids responsible for restraining HPA activity in the pregnant group is not known. One likely region however is the arcuate nucleus. β-endorphin cells in the arcuate nucleus are known to project to the PVN where the former have been shown to terminate on CRH neurones (Drolet *et al*, 2001). In late pregnancy (by day 21) there is an increase in the number of arcuate neurones expressing POMC and its protein product β-endorphin (personal communication with A.J. Douglas). Therefore these opioid neurones in the arcuate nucleus may play an important role in modulating CRH release in response to stress. Enkephalin is co-expressed with CRH in pPVN cells, however since very few of these neurones are activated in pregnant rats following stress it seems unlikely that opioids from this

source are acting to restrain CRH secretion. Opioids (such as enkephalins and dynorphins) are synthesised by neurones in the NTS (Bronstein *et al*, 1992; Ceccatelli *et al*, 1992) and retrograde labelling has shown that noradrenergic neurones project to the PVN (Ericsson *et al* , 1994). It is therefore conceivable that opioids may be co-expressed in the brainstem NTS noradrenergic neurones that innervate the PVN. If this is indeed the case, these neurones may be the same NTS neurones found here to be activated by IL-1β administration. Moreover, if these opioids are co-released with NA in response to immune challenge then it is plausible that in pregnancy the amount of opioids being released is increased, which may then act on the NA neurones themselves to limit NA release or on CRH neurones to inhibit their responses. Indeed pro-enkephalin (pENK) and μ-opioid receptor mRNA expression in brainstem NTS neurones has recently been shown to be significantly elevated in late pregnancy (Meddle *et al*, 2001).

Effect of opioids on oxytocin secretory responses to immune challenge

Endogenous opioids actively restrain oxytocin secretion during late pregnancy, and this may enable the posterior pituitary to accumulate oxytocin stores in preparation for parturition when large amounts of oxytocin are required for uterine contraction (reviewed in (Russell & Leng, 1998)). To test whether endogenous opioids were actively restraining oxytocin secretion in response to i.v. IL-1β, rats were pretreated with the opioid receptor antagonist, naloxone. Administration of naloxone evoked a modest, though insignificant increase in basal plasma oxytocin concentration in both the virgin and the pregnant rats. However naloxone pretreatment revealed a hyper-secretion of oxytocin in response to IL-1β in the pregnant rats. These data indicate that in pregnancy the oxytocin secretory response to immune challenge is restrained by endogenous opioids. A similar response has been observed in pregnant rats following exposure to forced swimming (Douglas *et al* , 1998; Neumann *et al*, 1998). Thus when opioid inhibition is blocked by naloxone an exaggerated oxytocin response is unmasked in the pregnant group. This hypersecretion of oxytocin in response to IL-1β may, in part reflect expanded neurohypophysial stores of oxytocin, however it may also be a consequence of increased drive to the oxytocin neurones

and/or enhanced opioid tone on either the oxytocin cells themselves or their excitatory inputs (e.g. noradrenergic nerve terminals). Indeed, evidence reported by Douglas *et al* would support this theory. They demonstrated that treatment with naloxone potentiates the increase in firing rate of oxytocin neurones in the SON induced by CCK in day 21 pregnant rats (but not in 16 day pregnant or virgin rats) (Douglas *et al* , 1995), indicating that increased activation of oxytocin cell bodies in response to excitatory stimuli is masked by enhanced opioid inhibition in late pregnancy. The functional significance of oxytocin release in response to IL-1β, as observed in the virgin group is not clear. Elevated levels of oxytocin following stress (but not basal oxytocin) have been reported to potentiate the ACTH response to exogenously administered CRH *in vivo* (Rivier & Vale, 1985), therefore oxytocin may be involved in modulating activity of the HPA axis in response to stress. However, the effects of oxytocin in the periphery, with regard to facilitating adaptive responses to stress are not clear. Oxytocin has been reported to increase both heart rate and blood pressure (Ishizuka *et al*, 1993) which may help to facilitate "fight-flight" responses. Oxytocin can also act on the thymus gland to enhance T cell growth (Geenen *et al*, 1989), which clearly may be important in response to immune challenge.

Summary and Conclusions

Thus in pregnancy the responsiveness of the HPA axis to immune challenge with LPS and IL-1β (physical stressors) is markedly attenuated, as it is following exposure to the emotional stressors described in Chapter 3. The reduction in ACTH and corticosterone secretion following IL-1β administration is probably a consequence of reduced drive by CRH neurones. The failure of the CRH neurones to respond to IL-1β in the pregnant rats is not a consequence of interrupted cytokine signalling to brainstem neurones in pregnancy, since the Fos study indicated that the NTS neurones were similarly activated by IL-1β in the virgin and pregnant groups. Thus the mechanisms that limit activation of the pPVN CRH neurones in response to immune challenge in pregnancy lie somewhere between the brainstem NTS neurones and the PVN. These mechanisms are likely to involve endogenous opioids, since

removing opioid inhibition in pregnancy reinstated an ACTH response and CRH mRNA response to IL-1β. Other mechanisms, not yet studied may involve changes in signalling from the NTS to the PVN via forebrain structures such as the CeA and the BNST (Buller *et al* , 2001).

Similarly the oxytocin secretory response to IL-1β is also significantly attenuated in pregnancy, however in this case pretreatment with naloxone, does not only reinstate an oxytocin response, instead it "unmasks" an exaggerated oxytocin response to IL-1β. The functional relevance of this strong opioid restraint over oxytocin secretion to stress in pregnancy, is probably concerned with expanding posterior pituitary stores for when they are required at parturition.

A distinctive pattern is beginning to emerge from the data presented in this and the previous chapter; this is that the HPA axis seems to be less responsive to both emotional and physical stressors in late pregnancy. Could it be that the HPA axis is less responsive to all stimuli in pregnancy? To address this question, the responsiveness of the HPA axis to metabolic signals is investigated in the next chapter.

CHAPTER 5

Effects of Orexin on the Hypothalamic-Pituitary-Adrenal Axis in Pregnancy

5.1. Introduction

The hypothalamus plays a major role in maintaining energy homeostasis by controlling food intake and body weight. Two main brain areas involved have been identified as the ventromedial hypothalamus (VMH), also termed the "satiety centre" and the lateral hypothalamus (LHA) or "hunger centre" (for review see (Bray *et al*, 1990)). Electrical stimulation of the VMH suppresses food intake, whereas stimulation of the LHA induces food intake. Bilateral lesioning of the VMH induces hyperphagia and obesity whereas lesioning of the LHA results in reduced food intake leading to anorexia.

5.1.1. Orexins

Three years ago, two novel hypothalamic peptides were identified and characterised. These neuropeptides were named orexin-A and orexin-B (from the Greek word for appetite, *orexis*) (Sakurai *et al*, 1998). Both orexin-A and orexin-B (also known as hypocretins 1 and 2) (De Lecea *et al*, 1998) have been shown to stimulate food intake (De Lecea *et al* , 1998; Sakurai *et al* , 1998), but they also have arousing actions.

Orexin-A is a 33-residue peptide and orexin-B is a 28-residue peptide. Both orexins are produced by the same 130 amino acid precursor protein, pre-pro-orexin, and share 46% amino acid homology. There are two types of orexin receptors, the orexin-1 (OX_1) receptor and orexin-2 (OX_2) receptor, which belong to the G-protein coupled receptor superfamily and share 64% sequence homology (Muhtashan *et al*, 2000). Orexin-A has highest affinity for the OX_1 receptor, while the OX_2 receptor binds both orexin subtypes, the affinity for orexin-B is 10-fold higher than for orexin-A (Muhtashan *et al* , 2000).

5.1.2. Distribution of Orexin and Orexin Receptors

In the rat brain, pre-pro-orexin mRNA and immunoreactive orexin-A expression is restricted to neurones within the lateral hypothalamus (LHA), a region classically implicated in the central regulation of feeding behaviour and energy homeostasis (Bernardis & Bellinger, 1993; Bernardis & Bellinger, 1996) and perifornical hypothalamus (Date *et al*, 1999), whereas orexin neuronal fibres are widely distributed throughout the brain, with widespread projections to the olfactory bulb, cortex, thalamus, hypothalamus and brainstem. Orexin containing nerve fibres are abundant in the paraventricular thalamic nucleus (PVT), dorsal raphe nucleus (DRN), central gray, median raphe nucleus. Moderate numbers of orexin fibres are also present in the PVN (excluding the lateral magnocellular division), locus coeruleus (LC), olfactory bulb, amygdala, suprachiasmatic nucleus (SCN), supramamillary nucleus, nucleus of the solitary tract (NTS) and the dorsal motor nucleus of the vagus (Date *et al* , 1999).

Messenger RNA for both orexin receptors is widely distributed throughout the brain. Within the hypothalamus OX_1 receptor mRNA is most abundant in the ventromedial nucleus (VMH), whereas OX_2 receptor mRNA is predominantly expressed in the PVN. These sites may be important for receiving orexigenic inputs from the LHA and the posterior hypothalamus since both the PVN and VMH have been implicated in feeding behaviour (Kalra *et al*, 1991).

5.1.3. Functions of Orexins

The majority of studies investigating the function of orexins have focussed on their physiological roles in feeding behaviour, energy homeostasis and the sleep/wake cycle. I.c.v. administration of either orexin-A or orexin-B stimulates food intake in a dose-dependent manner (Sakurai *et al* , 1998; Haynes *et al*, 1999; Edwards *et al*, 1999), while withdrawal of food for 48h hours causes up-regulation of hypothalamic pre-pro-orexin mRNA expression in rats (Sakurai *et al* , 1998). The effects of orexin-

A are longer lasting than those of orexin-B. Two hours after i.c.v. injection, orexin-B has little effect on stimulation of food intake, whereas the effects of orexin-A persist 4 hours after the injection (Sakurai *et al* , 1998). However both are significantly less potent in stimulating food intake when compared with neuropeptide Y (NPY; another appetite stimulating peptide) (Edwards *et al* , 1999).

The site at which orexins exert this effect is not known, however centrally administered orexin-A induces neuronal activation (indicated by *c-fos* mRNA expression) in the PVN, arcuate nucleus and the VMH (Edwards *et al* , 1999). The PVN has been reported to be an important area in regulation of food intake and is the major site of action for many orexigenic agents, including NPY (Stanley & Leibowitz, 1984) and galanin (Tempel *et al*, 1988), however little is known about the output pathways from the PVN involved in regulating food intake. Furthermore, the PVN and arcuate nucleus are the major sources of expression of endogenous NPY (Chronwall *et al*, 1985) and lesioning studies have indicated the VMH is an important area in regulating food intake (Bray, 1984). The presence of orexin receptors in these brain areas further implicates them as participating in the actions exerted by orexin on food intake. The wide distribution of orexin fibres suggests that orexins play an important role in other physiological functions besides feeding, indeed recently it has been demonstrated that orexins increase gastric acid secretion (Takahashi *et al*, 1999) as well as increasing blood pressure and heart rate (Samson *et al*, 1999). Other behaviours, besides feeding, have also been reported to increase following central administration of orexins, including face washing, grooming, burrowing and exploring (Ida *et al*, 1999). Furthermore, orexin has been reported to be involved in regulating the sleep-wake cycle (Hagan *et al*, 1999).

Narcolepsy is a neurological disease that affects both humans and animals. It is characterised by the sudden onset of sleep at times when it would not normally occur (eg. during the daytime in humans), uncontrollable bouts of rapid eye movement (REM) sleep and episodes of cataplexy (sudden and temporary paralysis) (Chemelli *et al*, 1999). Recent breakthroughs in canine narcolepsy demonstrated that a mutation in the orexin-2 receptor gene are the underlying cause behind the disease (Lin *et al*,

1999), suggesting a possible connection between orexins and regulation of the sleep-wake cycle. Orexin knockout mice also show symptoms of narcolepsy (Chemelli *et al* , 1999) and in human narcolepsy, there is a reduction in the number of orexin neurones in the brain (Thannickal *et al*, 2000). These findings that dysfunctional orexin receptors and the removal of orexin pathways both lead to narcolepsy emphasises that orexin pathways play an important role in arousal and sleep regulation. Thus, in addition to their effects on feeding behaviour, orexins are also believed to be involved in increasing arousal and locomotor activities.

5.1.3. Regulation of Appetite and Energy Homeostasis

The pancreatic hormone insulin, and leptin a hormone secreted by adipocytes, are the two key signalling hormones involved in regulating energy homeostasis. Both hormones circulate at levels proportional to body fat content (Bagdade *et al*, 1967; Considine *et al*, 1996) and enter the CNS in levels proportionate to their concentration in plasma (Baura *et al*, 1993; Schwartz *et al*, 1996b). Receptors for both leptin and insulin are present in brain areas involved in appetite/energy regulation (Baskin *et al*, 1988; Cheung *et al*, 1997; Baskin *et al*, 1999). Leptin and insulin act on specific neurones in the hypothalamus to stimulate neural circuits that inhibit food intake and increase energy expenditure and inhibit other neural circuits that increase food intake and reduce energy expenditure.

Besides orexins, several other hypothalamic neuropeptides have been implicated in controlling energy homeostasis (see table 5.1.).

Stimulates Feeding	Inhibits Feeding
Neuropeptide Y (NPY)	Pro-opiomelanocortin (POMC)
Agouti Related Peptide (AGRP)	α-Melanocyte Stimulating Hormone (α-MSH)
Orexins (A and B)	Corticotropin Releasing Hormone (CRH)
Melanin Concentrating Hormone (MCH)	Cocaine & Amphetamine Regulated Transcript (CART)
Galanin	Thyroid Releasing Hormone (TRH)
	Interleukin-1β (IL-1β)

Table 5.1. Neuropeptides involved in control of energy homeostasis

Neuropeptide Y (NPY) and Agouti related peptide (AGRP) are colocalised in a set of arcuate nucleus neurones (Hahn *et al*, 1998; Broberger *et al*, 1998). Pro-opiomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART) are colocalised in another set of arcuate nucleus neurones (Elias *et al*, 1998). These neurones are thought to provide an important pathway for leptin signalling to the brain. Indeed most NPY/AGRP and POMC/CART neurones coexpress leptin receptors (Cheung *et al* , 1997; Baskin *et al* , 1999) and are regulated by leptin in an opposing manner. NPY/AGRP neurones are inhibited by leptin (Stephens *et al*, 1995; Schwartz *et al*, 1996a; Hahn *et al* , 1998; Broberger *et al* , 1998), while POMC/CART neurones are stimulated by leptin (Thornton *et al*, 1997; Schwartz *et al*, 1997; Kristensen *et al*, 1998).

The PVN, PFA and LHA all receive rich innervation by axons from the arcuate nucleus NPY/AGRP and POMC/CART neurones (Elmquist *et al*, 1999). Orexin neurones are thought to be under the influence of these inputs: inhibited by the POMC/CART input and stimulated by the NPY/AGRP input from the arcuate nucleus.

5.1.3. Effects of Orexins on the HPA Axis

The HPA axis is involved importantly in metabolic regulation via the action of corticosterone on glucose mobilisation (Wahlestedt *et al*, 1987; Hanson & Dallman, 1995), and it is now clear that factors regulating appetite also affect HPA axis activity. It has recently been reported that i.c.v. administration of orexin-A elevates plasma ACTH and corticosterone in non-stressed rats (Jaszberenyi *et al*, 2000; Ida *et al*, 2000). This pituitary-adrenal activation is associated with the induction of *c-fos* mRNA in the parvocellular division of the PVN (pPVN) (Jaszberenyi *et al* , 2000), suggesting the involvement of orexins on HPA axis activity. Moreover, pretreatment of rats with the CRH antagonist, α-helical CRH has been shown to block the behavioural effects of centrally administered orexin-A (Ida *et al* , 2000) and completely abolish the corticosterone response evoked by orexin-A and orexin-B (Jaszberenyi *et al* , 2000). These data suggest that orexin-A may be acting centrally and that its effects are mediated via secretion of CRH. Al-Barazanji and colleagues recently reported that in addition to the induction of *c-fos* in the pPVN following i.c.v. injection of orexin-A, there is an increase in CRH and AVP mRNA expression in this region (Al-Barazanji *et al*, 2001). This finding further supports a role for CRH in mediating the action of orexin-A but also implicates a mediatory role for AVP.

In chapter three, it was suggested that the attenuated responsiveness of the HPA axis during pregnancy is likely to be a consequence of the CRH neurones or the pathways impinging upon them being less responsive to the stressors used. If indeed the CRH neurones are less responsive, it follows that they should also respond less to other stimuli. Since orexin-A has been shown to activate the HPA axis at a central level it provides a convenient model in which to study this hypothesis. Thus, the aim of the present study was to seek changes in the responsiveness of the HPA axis to centrally administered orexin-A during pregnancy.

5.2. Materials and Methods

5.2.1. Animals

Female Sprague Dawley rats were used throughout this set of experiments and were maintained under conditions detailed in chapter 2. After surgery all rats were caged individually.

5.2.2 Surgery

Five days prior to the day of the experiment, virgin and pregnant rats (day 16 of pregnancy) were fitted with jugular vein and intracerebroventricular (i.c.v.) cannulae under halothane anaesthesia, as described previously (see section 2.3. of chapter 2).

5.2.3. Experimental Procedure

On the morning of the experiment (between 07:30-09:00h), rats had the jugular vein cannula connected to PVC extension tubing (wall = 1mm, internal diameter = 0.5mm) filled with heparinised saline (1ml heparin; 5000 units/ml in 100ml 0.9% saline) and attached to a 1ml syringe. I.c.v. cannulae were prepared as follows. The cannula was attached to polythene tubing (wall = 0.5mm, internal diameter = 0.5mm) and then filled with ddH_2O. Using a Hamilton syringe, 2µl of either orexin-A (0.5µg or 140 pmoles orexin-A [Sigma]) or vehicle (artificial cerebrospinal fluid, aCSF) was drawn into the tip of the cannula and separated from the ddH_2O by a small air bubble. The dummy i.c.v. cannulae were removed and i.c.v. injection cannulae (containing either drug or vehicle) were inserted into the guide cannula. Rats were then left undisturbed for two hours, prior to the start of blood sampling.

Blood Sampling

In each case, 0.5 ml blood samples were withdrawn into 1ml syringes containing 50 µl of chilled 5% EDTA. Blood was stored in eppendorfs on ice until centrifugation

(see general methods). Two basal blood samples (0.5ml) were taken 30 minutes apart. Following the second basal sample, rats were treated with either 2µl orexin-A (0.5µg) or aCSF i.c.v., over a period of ~30 seconds. Sequential blood samples were then taken 10, 20, 30, 60, 90 and 120 minutes after the i.c.v. infusion. The volume of blood withdrawn was replaced with an equal volume of 0.9% sterile saline. Rats were killed by decapitation 4 hours after the i.c.v. infusion. Brains and brainstems were removed, frozen and stored as before (chapter 2).

5.2.4. *In situ* hybridisation

Brains were sectioned coronally at 15µm and mounted on slides as previously described in the general methods chapter. To detect CRH mRNA expression a 42-mer oligonucleotide probe was used [MWG-Biotech]. The sequence of the CRH mRNA oligo-probe used is given below. It is complementary to bases 496-537 which encode amino acids 22-35 of the rat CRH peptide (Jingami *et al*, 1985).

5'- CCT GTT GCT GTG AGC TTG CTG AGC TAA CTG CTC TGC CCT GCC-3'

Probe labelling and hybridisation were performed as previously described. The post-hybridisation washes were performed in the usual way. The melting temperature of the CRH mRNA oligo-probe is 78°C, therefore the heated SSC washes were performed at 58°C. Once dry, the sections were exposed to autoradiographic film for 21 days at room temperature. CRH mRNA expression in the PVN was quantified from autoradiographs using a computer based image analysis system as described in the general methods chapter.

5.2.5. Radioimmunoassays

Plasma ACTH and corticosterone were determined using commercially available kits (see sections 2.6.1. and 2.7.). The sensitivity was 1 pg/ml and 0.4 ng/ml, respectively and the intra-assay variation <11% and <6%, for the ACTH and corticosterone assays respectively.

5.2.6. Behavioural Measurements

The number of 'grooming behaviour events' was recorded during the two hours immediately following the i.c.v. injection. Face and head washing, body grooming, genital licking and tail cleaning were all included as measures of grooming behaviour for the purpose of this study.

5.2.7. Statistical Analysis

Two way repeated measures analysis of variance (2-way RM ANOVA), followed by Student-Newman-Keuls multiple comparison test was used to analyse plasma ACTH and corticosterone data. CRH mRNA was analysed using a two way ANOVA. Behavioural data were analysed using a Student t-test. P values less than 0.05 were considered statistically significant.

5.3. Results

5.3.1. Effects of i.c.v. orexin-A on plasma ACTH and corticosterone

Basal plasma concentrations of ACTH and corticosterone did not differ between any of the groups. I.c.v. administered aCSF had no significant effect on plasma ACTH in both the virgin and pregnant groups (figure 5.1.). However, the virgin rats treated with aCSF tended to display slightly higher levels of ACTH than the pregnant group, although this was not significant. I.c.v. infusion of orexin-A induced a rapid rise in plasma ACTH concentration in the virgin group ($p<0.001$; 2-way RM ANOVA), which was significantly higher than basal levels within 10 minutes of the infusion (71.2 ± 14.3 vs 22.4 ± 2.9 pg/ml), and remained elevated for 90 minutes (figure 5.1.). However, orexin-A failed to evoke any such effect on plasma ACTH in the pregnant group, with levels unchanged from pre-infusion values (figure 5.1). At 20, 30, 60 and 90 minutes after orexin-A administration, plasma ACTH concentration was

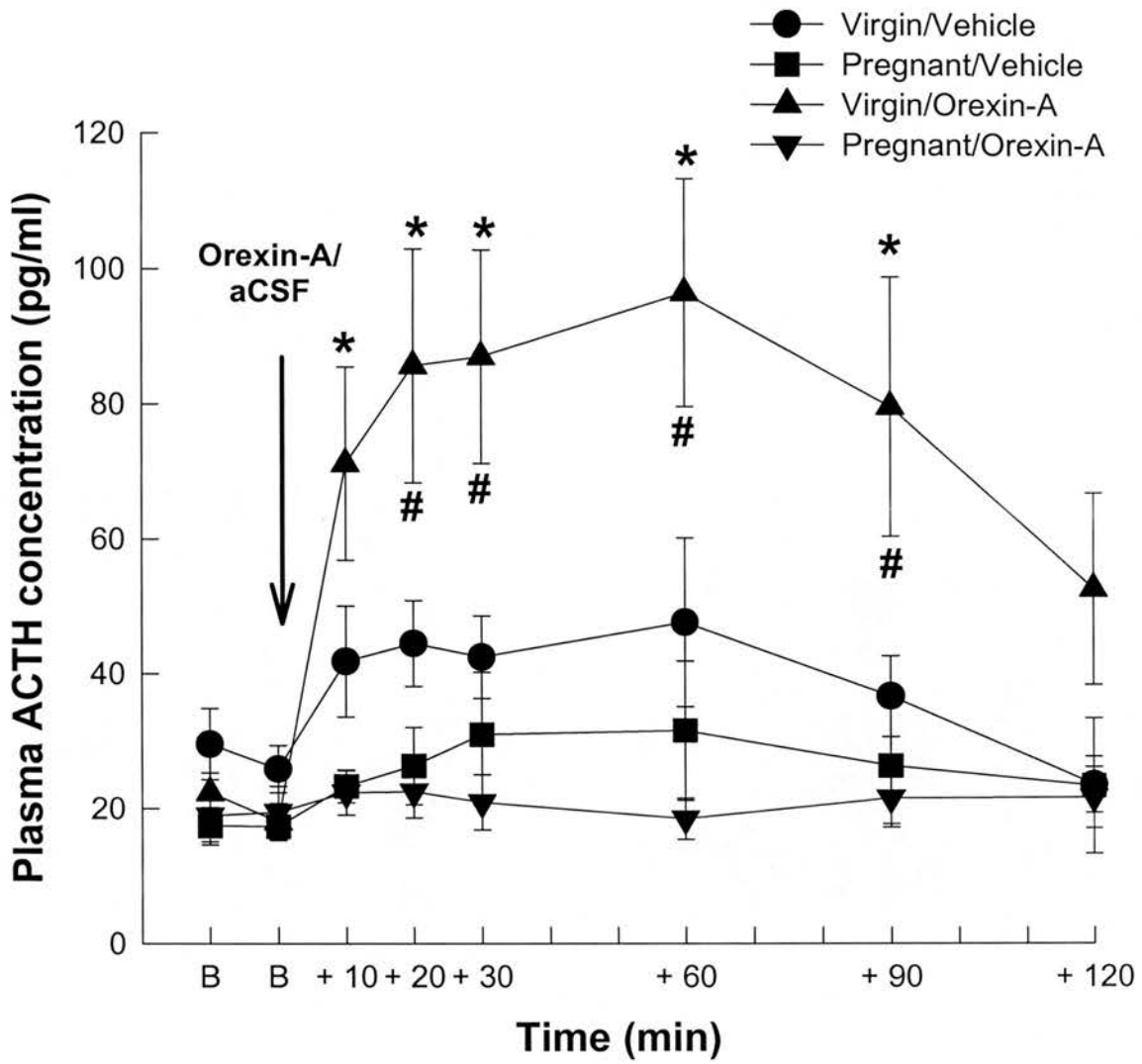


Figure 5.1. The effect of i.c.v. orexin-A on plasma ACTH concentrations in virgin and pregnant rats.

Two basal blood samples were collected 30 minutes apart, prior to i.c.v. administration of either aCSF or orexin-A (0.5μg). Further blood samples were withdrawn 10, 20, 30, 60, 90 and 120 minutes post-infusion. Values are plotted as group means ± SEM. Group numbers: virgin/vehicle, n = 5; pregnant/vehicle, n = 6; virgin/orexin-A, n = 5; pregnant/orexin-A, n = 8. Two-way ANOVA for repeated measures followed by student Newman-Keuls multiple comparison test was used for statistical analysis of the data: *$p < 0.001$ vs basal values in the same group; #$p < 0.002$ vs all other groups at the same time point.

significantly higher in the virgin group compared with the pregnant group. During this time plasma ACTH concentration in the virgin/orexin-A group was on average 4.2-fold higher than in the pregnant/orexin-A group.

Infusion of vehicle had no significant effect on plasma concentrations of corticosterone in either the virgin or the pregnant group (figure 5.2.). Administration of orexin-A induced a significant increase in corticosterone secretion only in the virgin group and had no effect in the pregnant rats (figure 5.2.). In the virgin group plasma corticosterone was significantly elevated from basal levels within 20 minutes of the orexin-A infusion (188.4 ± 46.3 vs 63.1 ± 9.7 ng/ml) and remained elevated at 120 minutes after the treatment.

5.3.2. Effects of i.c.v. orexin-A on CRH mRNA expression in the pPVN

Quantification of autoradiographs revealed that CRH mRNA expression in the pPVN of pregnant rats treated with aCSF was significantly less (by 78%) compared with the virgin group ($p < 0.001$; figure 5.3.). Orexin-A administered i.c.v. evoked a significant increase in CRH mRNA expression in the virgin group, however this response was absent in the pregnant group (0.408 ± 0.025 vs 0.095 ± 0.02, respectively; arbitrary units; figure 5.3.). The level of CRH mRNA expression in the PVN was 4.3-fold greater in the virgin/orexin-A group compared with the pregnant/orexin-A group (figure 5.3.).

7.3.3. Effects of i.c.v. orexin-A on grooming behaviour

I.c.v administration of aCSF had no significant effect on grooming behaviour in either of the groups (figure 5.4.). Administration of orexin-A into the lateral ventricle significantly increased grooming behaviour to a similar extent in both the virgin and the pregnant groups (4.5 ± 0.85 vs 3.5 ± 0.96 'grooming events'/ 60 min, respectively; $p< 0.03$; see figure 5.4.).

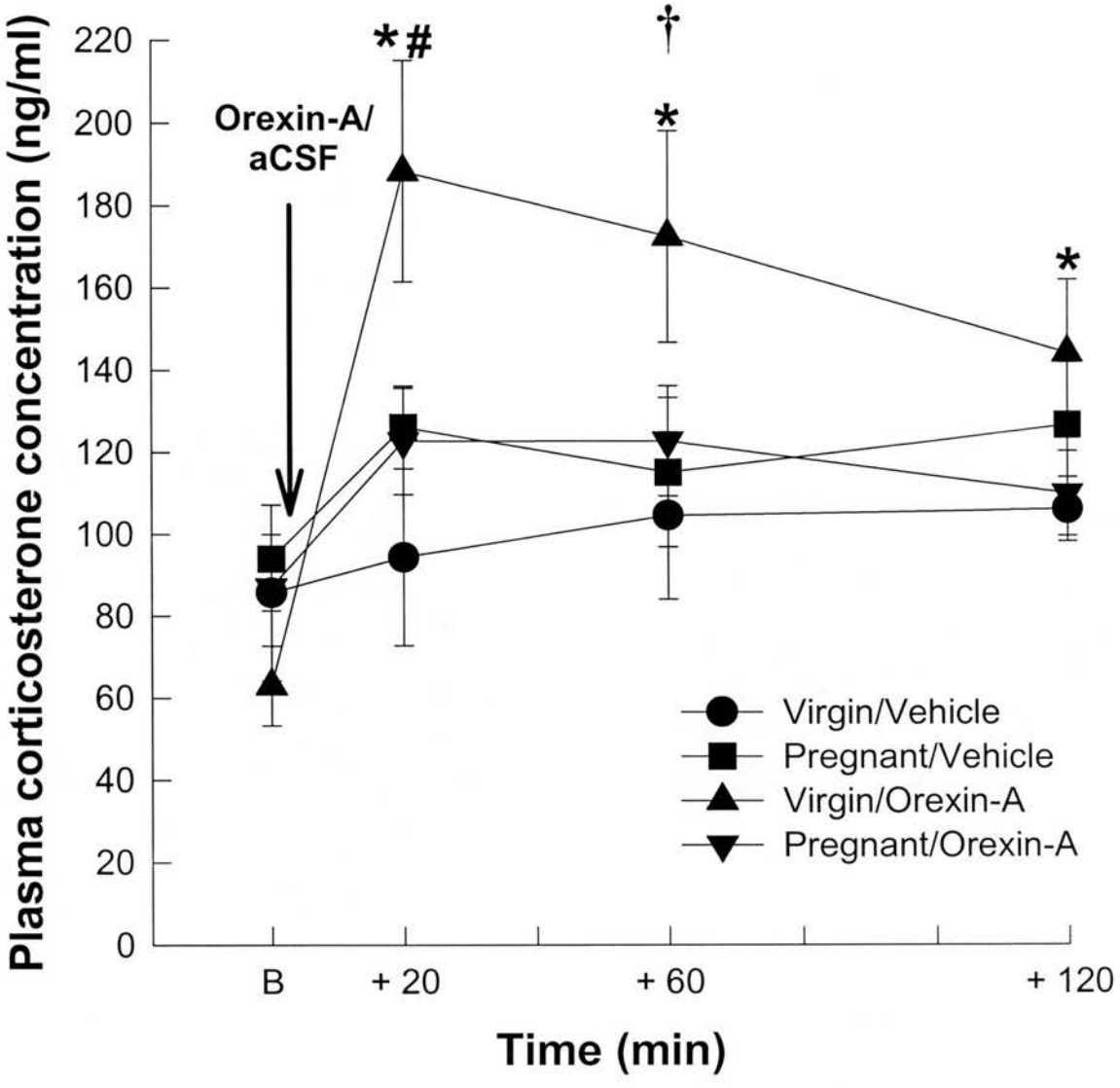


Figure 5.2. The effect of i.c.v. orexin-A on plasma corticosterone concentrations in virgin and pregnant rats.

One basal blood sample was collected before i.c.v. administration of either aCSF or orexin-A (0.5μg), followed by further blood samples 20, 60, 120 minutes post-injection. Values are plotted as group means ± SEM. Group numbers as before (see figure 5.1. legend). Two-way repeated measures ANOVA followed by Student Newman-Keuls multiple comparison test was used for statistical analysis of the data: *$p < 0.001$ vs basal values in the same group; †$p < 0.03$ vs all other groups at the same time point; #$p < 0.01$ vs all other groups at the same time point.

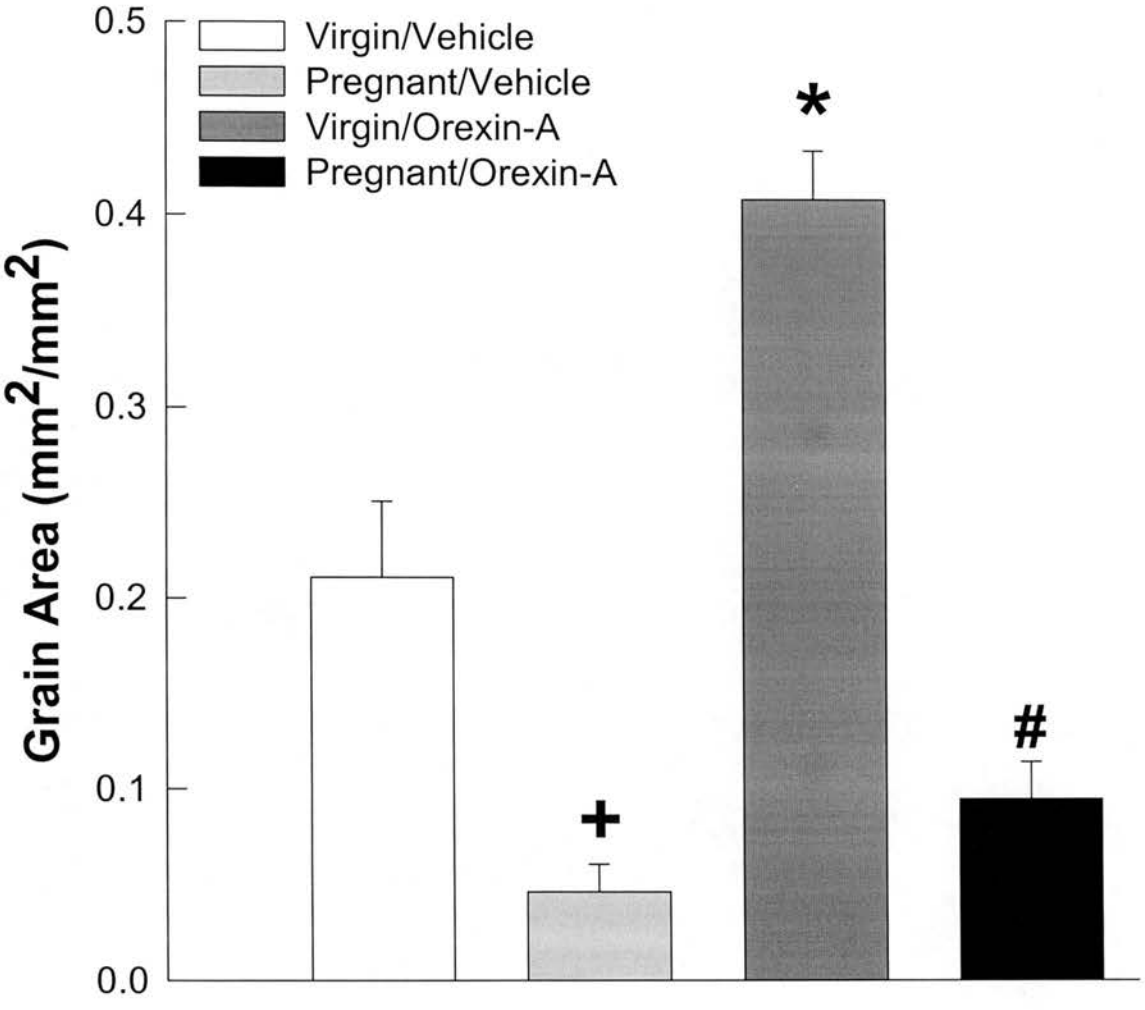


Figure 5.3.(a) The effect of i.c.v. orexin-A on CRH mRNA expression in the PVN of virgin and pregnant rats.

Rats were killed 4 hours after administration of either orexin-A (0.5μg) or aCSF. Coronal brain sections were hybridised with ^{35}S-labelled oligonucleotide probe for CRH mRNA. Autoradiographs were quantified by computer based image analysis. Values plotted are group means ± SEM for grain area (mm^2/mm^2). Group numbers: virgin/vehicle, n = 5; pregnant/vehicle, n = 8; virgin/orexin-A, n = 8; pregnant/orexin-A, n = 9. Two-way ANOVA followed by student Newman-Keuls multiple comparison test was used for statistical analysis of the data: +$p < 0.001$ vs virgin/vehicle group; *$p < 0.001$ vs all other groups; #$p < 0.001$ vs virgin/orexin-A group.

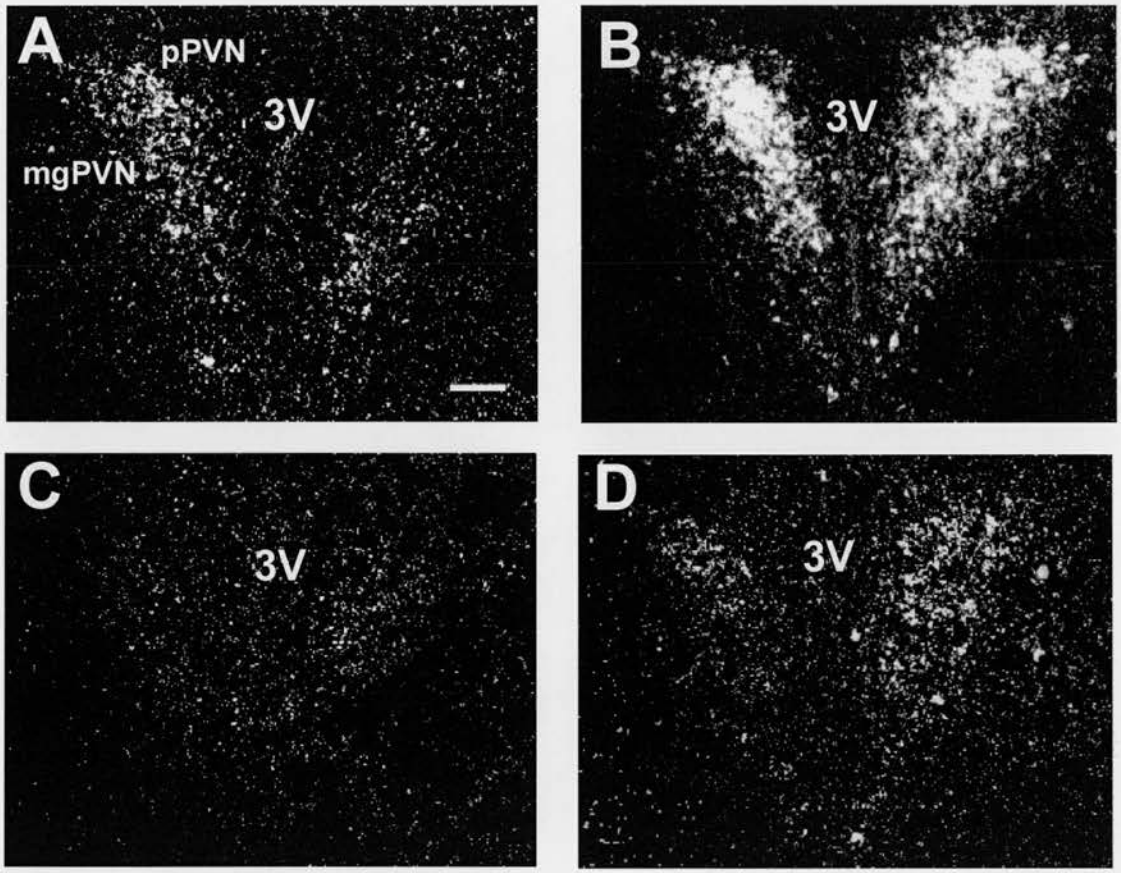


Figure 5.3.(b) The effect of i.c.v. orexin-A on CRH mRNA expression in the PVN of virgin and pregnant rats: Photomicrographs.

Darkfield autoradiographs of coronal sections through the paraventricular nucleus hybridised with a ^{35}S-labelled oligonucleotide probe complementary to corticotropin releasing hormone from: A, virgin/vehicle; B, virgin/orexin-A; C, pregnant/vehicle; D, pregnant/orexin-A. 3V, third ventricle; mgPVN, magnocellular division of PVN; pPVN, parvocellular division of PVN. Scale bar: 100μm

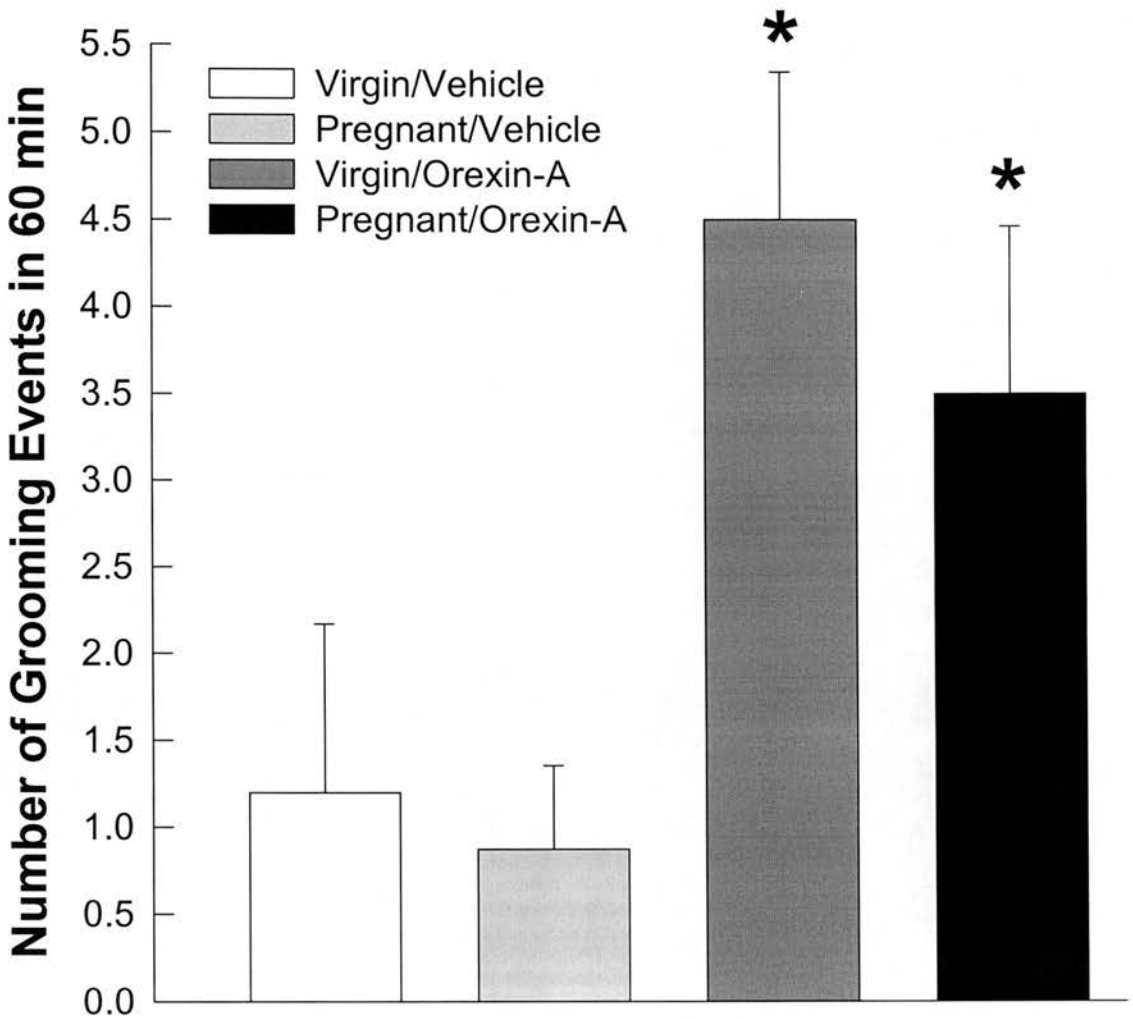


Figure 5.4. The effects of i.c.v. orexin-A on grooming behaviour in virgin and pregnant rats.

Grooming behaviour was recorded after administration of either i.c.v. aCSF or orexin-A. Values are plotted as group means ± SEM. Group numbers as before (see figure 5.3. legend). Student's t-test was used to analyse the data: *$p < 0.03$ vs vehicle treated group.

5.4. Discussion

The present study has demonstrated that i.c.v. administration of orexin-A evokes HPA axis activation in conscious virgin female rats, consistent with previous studies in male rats (Jaszberenyi *et al* , 2000; Al-Barazanji *et al* , 2001). Here the marked increase in plasma ACTH and corticosterone secretion following treatment with orexin-A was associated with increased expression of CRH mRNA in the parvocellular region of the PVN 4 hours after the injection. This is in agreement with a study which demonstrated increased *c-fos* mRNA in this region (Kuru *et al*, 2000) and another recent study which reported increased CRH and AVP mRNA expression in the pPVN (Al-Barazanji *et al* , 2001). However in late pregnant rats, i.c.v. orexin-A failed to evoke any such increase in plasma ACTH and induced only a non-significant rise in corticosterone secretion. Furthermore, orexin-A did not have any significant effect on CRH mRNA expression in the PVN of pregnant rats. CRH mRNA levels were significantly attenuated in pregnant rats treated with aCSF compared with their virgin counterparts. It is possible that injecting fluid into the brain (albeit vehicle) induces a small stress response, which is attenuated in pregnancy or that these levels of CRH mRNA expression may reflect basal levels. This would be consistent with findings reported by Johnstone *et al* (Johnstone *et al*, 2000a) which demonstrated attenuated levels of CRH mRNA expression under basal conditions on day 21 of pregnancy. This may be a consequence of reduced neural drive or enhanced inhibitory inputs to the CRH neurones at the end of pregnancy.

Orexin-A is considered to exert its effects on the HPA axis by acting centrally at the level of CRH and/or AVP neurones in the PVN (Jaszberenyi *et al* , 2000; Al-Barazanji *et al* , 2001), although the mechanisms of its actions are not clear (some of the possibilities are shown in figure 5.5). The present data would support this, in that the CRH neurones are clearly activated by orexin-A in non-pregnant rats. The PVN is innervated by orexin-A immunoreactive projections which originate from orexin cell bodies in the LHA and PFA (Cutler *et al*, 1999). OX_2 receptor mRNA is predominately expressed in the PVN region (Trivedi *et al*, 1998) suggesting that if

the activation of the HPA axis by orexin-A as reported here in virgin females and by others in male rats (Jaszberenyi *et al* , 2000; Al-Barazanji *et al* , 2001) is a consequence of a direct action of orexin-A at the level of the PVN it is likely that the orexin type 2 receptor mediates the response (although this cannot be confirmed until co-localisation of CRH neurones with orexin type 2 receptors has been demonstrated).

The effects of orexins on HPA axis hormone secretion in non-pregnant animals strongly resemble those of NPY (Tsagarakis *et al*, 1989b; Suda *et al*, 1993). A recent study demonstrated that pretreatment with either a NPY antagonist or NPY antiserum inhibits the corticosterone secretory response to orexin-A (Jaszberenyi *et al*, 2001). These data strongly suggest that NPY mediates orexin-induced activation of the HPA axis. Orexin-containing nerve terminals have previously been shown to synapse on NPY neurones in the arcuate nucleus (Peyron *et al*, 1998; Horvath *et al*, 1999) and NPY projections from the arcuate nucleus to the PVN are abundant (Liposits *et al*, 1988; Broberger *et al*, 1999; Horvath *et al* , 1999; Li *et al*, 2000). Thus it may be hypothesised that under conditions of fasting, orexin neurones in the LHA are activated, by signals from the periphery (as a result of decreased leptin and insulin). Activation of these neurones triggers orexin release, which binds to orexin receptors on NPY neurones in the arcuate nucleus. These activated neurones release NPY in the PVN inducing activation of parvocellular CRH neurones and consequently the HPA axis. Here orexin-A was administered i.c.v., meaning it may be acting anywhere in the brain, so whether orexin is exerting its effects on the HPA axis via a mediatory action of NPY, or acting directly via its actions on type 2 receptors in the PVN or via its receptors on brainstem NTS neurones is not known (see figure 5.5).

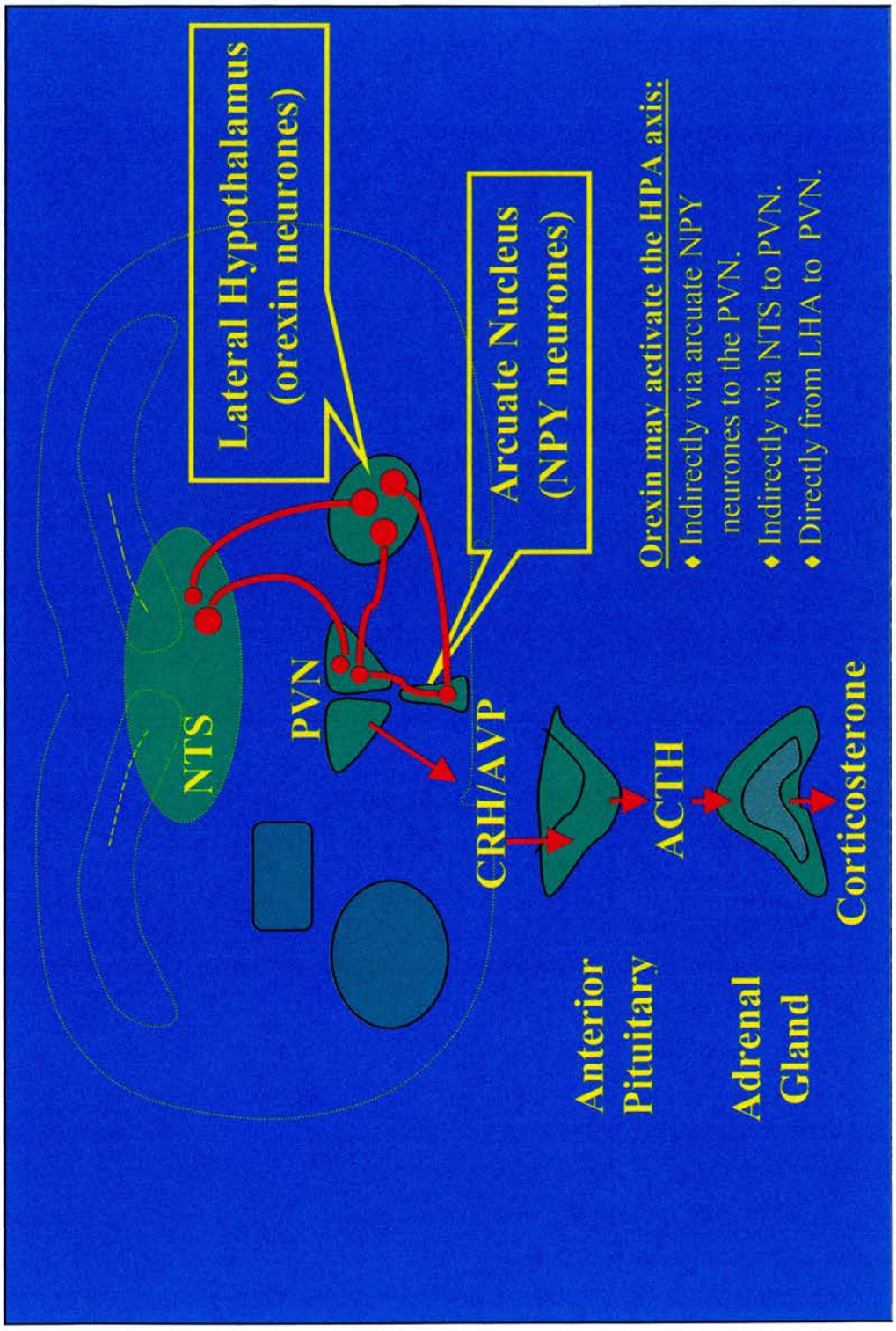


Figure 5.5 Possible mechanisms involved in activation of the CRH neurones by orexin.

Since it is unclear how orexin activates the HPA axis in non-pregnant rats, it is difficult to predict what adaptations are occur in this signalling pathway in pregnancy that prevent CRH neurones responding to orexin-A. To date, nothing is known about orexin receptor expression in pregnancy, however if the orexin receptors on the NPY neurones in the arcuate nucleus or orexin receptors in the PVN itself were down-regulated in pregnancy or blocked by an endogenous antagonist, then it would be expected that orexin would be incapable of activating CRH neurones. Alternatively, NPY receptors on PVN CRH neurones may be down-regulated in pregnancy preventing activation of CRH neurones mediated via NPY.

The activity of CRH neurones projecting to the median eminence may provide information about centrally projecting CRH neurones. The PVN CRH neurones that project to the median eminence are non-responsive to stimulation by orexin-A in pregnancy. Since centrally acting CRH is known to inhibit food intake (Schwartz *et al*, 2000), it may be that CRH neurones that project to feeding centres in the brain are also non-responsive in pregnancy. It would be beneficial to increase appetite in pregnancy so as to help meet the demands of the fetuses while maintaining a positive energy balance in the mother. However the behavioural data suggest that this may not be the case. The experiment was performed in the morning during the light phase, a time when rats are normally inactive. At the beginning of the experiment most rats were sleeping/resting. Those given vehicle were found to become active briefly before settling down again, whereas the rats injected with orexin-A became more active. Orexin-A administration significantly increased the incidence of grooming behaviour similarly in both virgin and pregnant rats, and although not measured, orexin treated rats seemed to demonstrate increased 'oral motor activity'. The fact that orexin-A induced grooming behaviour at a time when rats are normally inactive, supports a role for orexin as an arousing peptide. Grooming behaviour is thought to be induced by increased central release of CRH (Dunn *et al*, 1988). So, if centrally projecting neurones were also less responsive to stimulation by orexin-A in pregnancy, we may expect central administration of orexin-A to have little effect on grooming behaviour in the pregnant group.

However, no difference in grooming behaviour was observed between the orexin treated virgin and pregnant rats. It may be that CRH is not the sole factor involved in orexin-A induced grooming behaviour or that CRH pathways to feeding centres are differentially regulated from those involved in eliciting grooming behaviour.

This study has clearly demonstrated that the responsiveness of the HPA axis (at all levels) to the appetite stimulating/arousing peptide, orexin-A is attenuated in late pregnancy. Taken together with the data from the experiments employing 'psychological' and 'physical' stressors in chapters 3 and 4 respectively, it seems that in pregnancy an adaptation (or adaptations) occurs which results in a 'global' attenuation in the responsiveness of the HPA axis to a variety of stimuli. Collectively the data support the theory that it is indeed the CRH neurones themselves that are less or even non-responsive. This adaptation seems to occur by some unknown mechanism activated in late gestation and is evident irrespective of the nature of the stressor applied. The possible mechanisms involved will be explored later.

CHAPTER 6

The role of glucocorticoid negative feedback in attenuated hypothalamo-pituitary-adrenal axis responses to stress in pregnancy

6.1. Introduction

There are several possible adaptations that may explain the hyporesponsiveness of the HPA axis to stress in pregnancy. Changes at the level of the anterior pituitary are likely to be involved, since ACTH secretion is less sensitive to systemically administered CRH in late pregnancy and the number of CRH binding sites is significantly reduced from mid-gestation onwards in the rat (Neumann *et al*, 1998). Attenuated central drive to the axis may also explain the hyporesponsiveness. Reduced *c-fos* mRNA expression has been demonstrated in the PVN and in afferent processing areas such as the medial amygdala and lateral septum (da Costa *et al*, 1996) in response to acute restraint stress in late pregnancy, indicating diminished activation of the neurones in response to stressors.

In chapters 3-5 the role(s) of attenuated forward drive to the PVN neurones and less-responsive CRH neurones in explaining the hyporesponsiveness in late pregnancy was discussed. As well as changes in afferent inputs to the PVN and reduced sensitivity of CRH neurones to stress in pregnancy, changes in the glucocorticoid feedback signal may also influence the responsiveness of the HPA axis to stressors. As mentioned earlier (Chapter 1) the stress axis is sensitive to feedback inhibition by corticosterone under basal and stress conditions, at the level of the anterior pituitary, hypothalamus and other higher centres (see Chapter 1). Corticosterone exerts its negative feedback effects through two different types of receptor in the CNS, mineralocorticoid (MR) and glucocorticoid (GR) receptors. MR mRNA has limited distribution, being restricted to the hippocampus and lateral septum (Reul & De Kloet, 1985), whereas GR mRNA expression is more widespread and found in the hippocampus, amygdala, hypothalamus and brainstem. MRs are ~90% occupied during the normal circadian nadir and are thought to be involved in controlling basal expression of CRH and AVP (Dallman *et al*, 1987; Dallman *et al*, 1994).

The finding that GRs become occupied in the presence of elevated corticosterone levels (Reul & De Kloet, 1985) such as after exposure to stress, suggests that these receptors play an important role in regulating stress induced ACTH secretion.

The effects of corticosterone negative feedback on the HPA axis have been well described. Adrenalectomy results in a rapid hypersecretion of ACTH (Dallman *et al*, 1972) with a corresponding increase in POMC mRNA expression in the anterior pituitary (Jingami *et al*, 1985a). In the parvocellular PVN, CRH and AVP mRNA expression increases (Jingami *et al* , 1985a), as does CRH and AVP peptide expression. The mechanisms by which glucocorticoids effect feedback on CRH and AVP gene transcription are unclear, but are thought to involve either a direct interaction between an occupied GR and a glucocorticoid response element (GRE) in the promoter region of the AVP gene (Mohr & Richter, 1990; De Kloet *et al*, 1998) or an indirect mechanism via transcription factors which switch off transcription (Bamberger *et al*, 1996).

Changes in basal ACTH and corticosterone secretion are apparent during pregnancy. The circadian rise in ACTH secretion diminishes after mid-gestation. Daily corticosterone levels decline in early pregnancy, reaching their lowest levels by day 10 (Atkinson & Waddell, 1995), however in the second half of pregnancy (~ day 14) corticosterone secretion increases progressively as term approaches, with no concomitant rise in ACTH secretion (Atkinson & Waddell, 1995), which probably reflects changes in the sensitivity of the adrenal gland to ACTH, possibly in response to increasing levels of oestrogen. It is not clear whether this increase in corticosterone secretion (the daily mean is 34% higher on day 22 of pregnancy than in non-pregnant females) will necessarily increase the negative feedback signal, since CBG levels have been shown to increase in pregnancy (Lohrenz *et al*, 1967), which may limit the amount of free circulating corticosterone. Basal expression of CRH and AVP mRNA in the pPVN is attenuated by day 21 of pregnancy (Johnstone *et al*, 2000), however whether this is a result of enhanced negative feedback or a consequence of reduced central drive to these neurones has not been established. Nevertheless, evidence for enhanced feedback mechanisms has been reported in

pregnancy. Activity of 11β-HSD-1 (which predominately acts as a reductase in intact cells to reactivate corticosterone from inert 11-dehydrocorticosterone) has been reported to increase in the PVN during the second half of pregnancy (Johnstone *et al* , 2000), which would presumably increase local glucocorticoid levels. Furthermore, levels of GR mRNA expression in the dentate gyrus have been shown to be significantly higher on day 21 than on day 10 of pregnancy (Johnstone *et al* , 2000), suggesting increased sensitivity to corticosterone negative feedback at this time. However changes in GR expression and 11β-HSD activity in pregnancy seem more likely to represent a mechanism for enhanced 'slow' negative feedback. In fact pregnant rats may be less sensitive to 'rapid' feedback since acute corticosterone replacement is less effective in decreasing ACTH in phADX pregnant rats compared with virgins (Johnstone *et al* , 2000). To date, the effects of rapid negative feedback by corticosterone on stress-induced ACTH secretion has not been investigated in pregnancy.

The present set of experiments was designed to investigate whether glucocorticoid negative feedback is enhanced in late pregnancy. Two approaches were employed to investigate this hypothesis. The first was to remove the glucocorticoid feedback signal. It was not feasible to do this in the traditional way by surgically removing the maternal adrenal glands, since the foetal adrenals are also known to secrete corticosterone (from ~ day 18 of pregnancy) which can cross the placenta and enter the maternal circulation (although this should be limited by placental 11β-HSD (Burton & Waddell, 1994)). Therefore synthesis of corticosterone was blocked pharmacologically, by administration of a series of metyrapone (a 11β-dehydroxylase inhibitor which prevents the conversion of 11-deoxycorticosterone to corticosterone) injections and a single injection of aminoglutethimide (an inhibitor of 20α-hydroxylase which prevents the conversion of cholesterol to pregnenolone) using a previously described protocol (Plotsky *et al*, 1986; Plotsky & Sawchenko, 1987).

In experiment 1, virgin and pregnant rats were treated over a 48h period to induce pharmacological adrenalectomy (phADX) and the effects of removing glucocorticoid

feedback on basal HPA activity were investigated. In the second experiment, rats were again treated to induced phADX and this time the effects of blocking corticosterone negative feedback on stress-induced responses were investigated. In the final experiment, rats were pretreated with corticosterone, prior to exposure to forced swimming, and the effects of enhanced rapid corticosterone negative feedback on stress responses were measured in virgin and pregnant rats.

The aims of each experiment were:
Experiment 1: to determine whether 'slow' glucocorticoid negative feedback control over basal HPA activity was enhanced in late pregnancy.
Experiment 2: to determine whether glucocorticoid negative feedback control over stress-induced HPA activity was enhanced in late pregnancy.
Experiment 3: to determine whether 'rapid' glucocorticoid feedback was more effective in restraining stress-induced HPA activity in late pregnancy.

6.2. Materials and Methods

6.2.1. Animals

Female Sprague Dawley rats were used throughout this set of experiments (weighing between 260-300g at the beginning) and were maintained under conditions detailed in chapter 2. For experiment 1, rats were housed singly for at least 5 days prior to the first experiment and for experiments 2 and 3, all rats were caged individually after surgery.

6.2.2 Surgery

For experiments 2 and 3, virgin and pregnant rats (day 16 of pregnancy) were fitted with a jugular vein cannula five days prior to the day of the experiment, under halothane inhalation anaesthesia, as described previously (see section 2.3. of Chapter 2).

6.2.3. Experimental Procedure

(i) Pharmacological Adrenalectomy

For experiments 1 and 2 it was necessary to block the synthesis of endogenous glucocorticoids. This was achieved pharmacologically by administration of the 11β-hydroxylase inhibitor, metyrapone [Sigma] and the 20α-hydroxylase inhibitor, aminoglutethimide [Sigma], as previously described (Plotsky *et al* , 1986; Plotsky & Sawchenko, 1987; Johnstone *et al* , 2000).

Experiment 1: Virgin and pregnant rats were treated with either 100 mg/kg metyrapone (dissolved in 0.9% saline) or saline subcutaneously (s.c.) at 8 hour intervals over a 48 hour period. Pharmacological adrenalectomy over 48h was chosen since a previous study has shown that there is a pronounced increase in CRH and AVP immunoreactivity in the PVN between 24-72 h of phADX treatment (Plotsky & Sawchenko, 1987). All rats in experiment 1 were given a total of seven metyrapone or saline injections, beginning at 08:00h on day 19 of pregnancy). The final metyrapone injection (at 08:00h on day 21 of pregnancy) was followed 75 min later by a subcutaneous injection of 200 mg/kg aminoglutethimide or vehicle (dimethyl-sulfoxide; DMSO)[Sigma]. One hour after the aminoglutethimide/DMSO injection rats were killed by decapitation. Brains were rapidly removed and frozen on dry ice for *in situ* hybridisation. Trunk blood was collected into chilled plastic tubes containing 5% EDTA and plasma was separated by centrifugation, as before (see general methods chapter) for determination of plasma hormone and glucose concentrations. The number of fetuses *in utero* was recorded and the combined weight of five pups (with placentae attached) was measured. Since glucocorticoids influence the weight of the thymus and the weight of the adrenal glands is influenced by ACTH, they were removed from the rats post-mortem and weighed to provide indicators of the success of adrenalectomy.

Experiment 2: Virgin and pregnant rats were treated as described in experiment 1, to induce a state of pharmacological adrenalectomy (phADX), however this time rats were given only 4 metyrapone/vehicle injections over a 24 hour period, beginning at 08:00h on day 20 of pregnancy. Here rats were treated to induce phADX over a 24h period as this has previously been shown to be sufficient time to reduce corticosterone secretion to minimal levels and hence induce increased ACTH secretion. Immediately before the final metyrapone injection (at 08:00 h on day 21), rats had extension tubing attached to a 1ml syringe (both filled with heparinised saline) connected to the jugular vein cannula. 75 min after the final metyrapone injection rats were administered either aminoglutethimide or DMSO as before.

(ii) Blood Sampling

Experiment 2: Jugular vein cannulae were connected at 07:00h on the day before the experiment (day 20 of pregnancy) and a blood sample was withdrawn approximately one hour later, prior to any treatment with metyrapone. After the blood sample was taken the cannula was flushed with heparinised saline and disconnected until the following morning. On the morning of the experiment (day 21 of pregnancy) cannulae were connected for blood sampling as described above. Two basal blood samples were withdrawn (30 min apart) 1 hour after the aminoglutethimide injection. Immediately after the second basal sample, all rats were transferred from their home cage into a Perspex restraining tube (diameter = 70 mm, the length of the tube was adjusted to prevent the animal from turning around) for 30 minutes and then returned to their home cage. Further blood samples were withdrawn 15, 30, 45, 60 and 90 minutes after the onset of the period of restraint. Rats were killed by an i.v. overdose of pentobarbitone and the number of fetuses *in utero* was recorded.

Experiment 3: On the morning (between 07:30-09:00h) of the experiment (day 21 of pregnancy) rats had the jugular vein cannula connected to PVC extension tubing (wall = 1mm, internal diameter = 0.5mm) filled with heparinised saline (1ml heparin, 5000 units/ml in 100ml 0.9% saline) and attached to a 1ml syringe. Rats were then left undisturbed for at least one hour prior to the start of blood sampling. Two basal

blood samples were withdrawn, 30 min apart. After the second basal sample, rats were administered either 2 mg/kg corticosterone-21-acetate [Sigma] s.c. (dissolved in a 10% alcohol in 0.9% saline solution) or vehicle. One hour later a further blood sample was withdrawn. Immediately after this blood sample was collected all the rats were forced to swim for 90 seconds in a cylindrical container (dimensions = height, 38 cm; diameter, 33 cm) filled to a depth of ~ 30cm with tap water at 19°C. After exposure to forced swimming, rats were gently towel-dried for 15-20 seconds and then returned to their home cages. Further blood samples were withdrawn 5, 15, 50 and 80 min after the end of the swim stress. After the last blood sample rats were killed by an i.v. overdose of pentobarbitone and the number of fetuses *in utero* was recorded.

The volume of blood withdrawn in each of the experiments detailed above was dependent upon the eventual assay. For analysis of ACTH only, 0.5 ml samples were taken, for ACTH and corticosterone, 0.7 ml and for corticosterone only 0.3ml samples were taken. All blood samples taken for analysis of ACTH and/or corticosterone were collected into 1 ml syringes containing 5% EDTA (see General Methods; Chapter 2). The volume of blood withdrawn was replaced immediately by an equal volume of 0.9% sterile saline. In each case blood was stored in eppendorfs on ice until centrifugation (see General Methods).

6.2.4. *In situ* hybridisation

Brains for *in situ* hybridisation were sectioned coronally at 15μm through the PVN and thaw-mounted on gelatinised slides as previously described in the General Methods (Chapter 2). To detect CRH mRNA expression a 42-mer oligonucleotide probe was used [MWG-Biotech]. The sequence of the CRH mRNA oligo-probe used is given below. It is complementary to bases 496-537 which encode amino acids 22-35 of the rat CRH peptide (Jingami *et al*, 1985b).

5'- CCT GTT GCT GTG AGC TTG CTG AGC TAA CTG CTC TGC CCT GCC -3'

To detect AVP mRNA expression a 45-mer oligonucleotide probe [MWG-Biotech], complementary to the sequence encoding the last 15 amino acids of the AVP peptide was used (Ma *et al*, 1997). The sequence is given below.

5'- GAC CCG GGG CTT GGC AGA ATC CAC GGA CTC TTG TGT CCC AGC CAG -3'

Probe labelling and hybridisation were performed as described in the General Methods (Chapter 2). The post-hybridisation washes were performed in the usual way. The melting temperatures of the CRH and AVP mRNA oligo-probes were 78°C and 81°C respectively, therefore the heated SSC washes were performed at 58°C and 61°C, respectively. Once dry, the sections were exposed to autoradiographic film: 21 days for CRH mRNA and 11 days for AVP mRNA (both at room temperature).

CRH and AVP mRNA expression in the parvocellular region of the PVN was quantified from autoradiographs using a computer based image analysis system (NIH Image 1.62) as described in the General Methods (Chapter 2).

6.2.5. Radioimmunoassays

All samples from a particular experiment were assayed together. Plasma ACTH and corticosterone concentrations were determined using commercially available kits (see sections 2.6.1. and 2.7.). The sensitivity was 1 pg/ml and 0.85 ng/ml, respectively and the intra-assay variation <10% and <6%, for the ACTH and corticosterone assays respectively.

6.2.6. Plasma Glucose Measurements

Since glucocorticoids play an important role in mobilising glucose stores, adrenalectomy can have major metabolic consequences for the rat. To ascertain whether the pharmacological adrenalectomy procedure was causing the rats to suffer any metabolic stress, plasma glucose levels were measured.

Plasma glucose concentrations were determined using test strips and a Reflolux® blood glucose meter [both from Boehringer Mannheim]. The principle of the test is based upon the glucose-oxidase/peroxidase reaction and the manufacturers have determined it is specific for glucose. The detection range of the test strips used was 0.5-27.7 mmol/l.

6.2.7. Statistical Analysis

Experiment 1: Levels of CRH and AVP gene expression and hormone levels in trunk blood were all analysed using a two-way ANOVA followed by a Student Newman-Keuls multiple comparison test. Plasma glucose concentrations were analysed using a Kruskal-Wallis analysis followed by Dunn's post-hoc test. Comparisons of adrenal and thymus gland weights were made using a two-way ANOVA. The effect of phADX on foetal weight was analysed using a two-tailed unpaired t-test.

Experiment 2: Two way repeated measures analysis, followed by Student-Newman-Keuls multiple comparison test was used to analyse plasma ACTH and corticosterone data. Plasma glucose concentrations were analysed using a two-tailed paired t-test.

Experiment 3: ACTH and corticosterone data from the blood sampling were analysed using a two-way ANOVA for repeated measures.

In each case, p values less than 0.05 were considered statistically significant.

6.3. Results

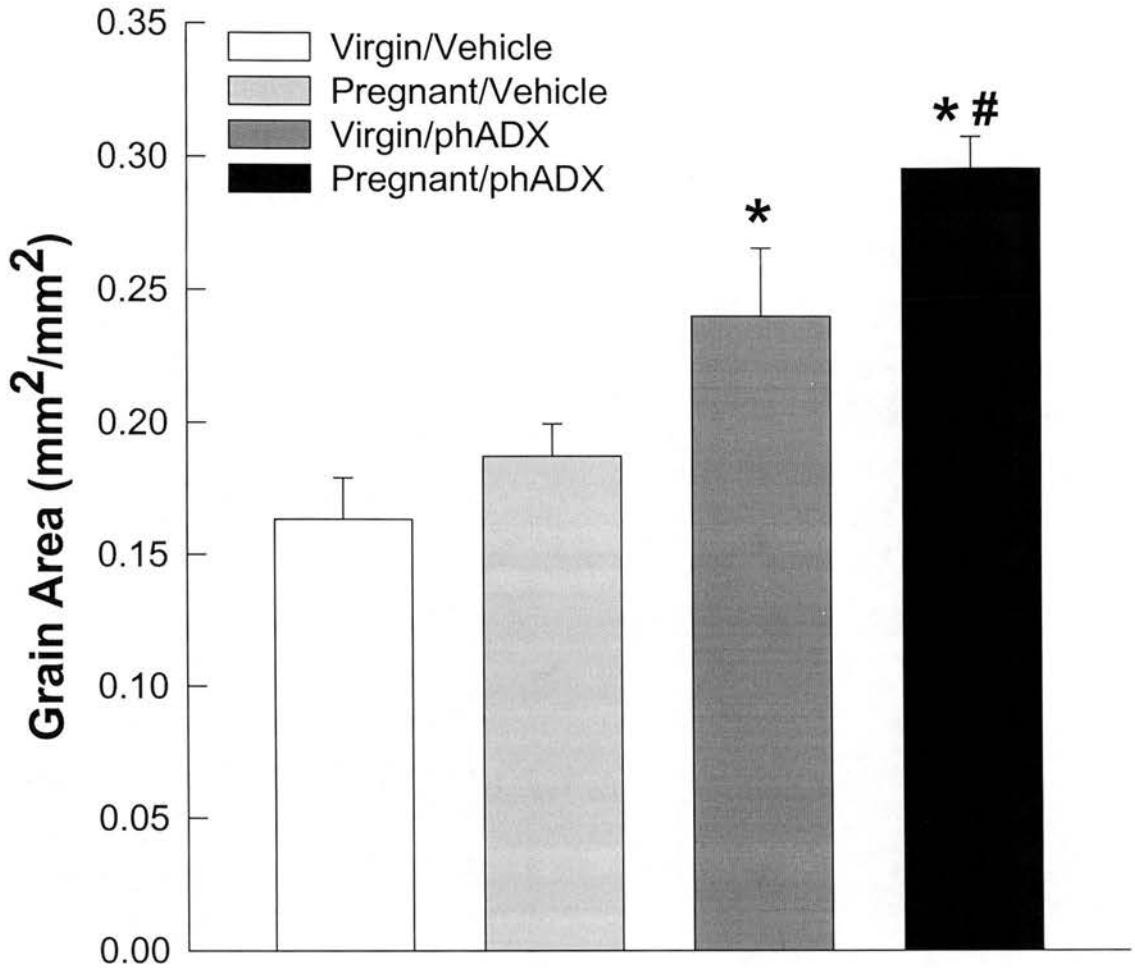
Experiment 1:Effect of 48h pharmacological adrenalectomy on basal HPA activity

6.3.1. Effect of 48h pharmacological adrenalectomy on basal expression of CRH mRNA in the pPVN

Quantification of film autoradiographs revealed that levels of CRH mRNA expression in the pPVN in vehicle treated rats was not significantly different between the virgin and the pregnant groups (figure 6.1.). Pharmacological adrenalectomy (phADX) for 48h induced a significant increase in the level of CRH mRNA expression in both the virgin and the pregnant groups ($p<0.05$; two-way ANOVA). The increase in CRH gene expression induced by metyrapone/aminoglutethimide treatment was significantly greater in the pregnant group (58 % increase from vehicle injected rats) compared with the virgin group (47 % increase from vehicle injected rats) ($p<0.05$; two-way ANOVA).

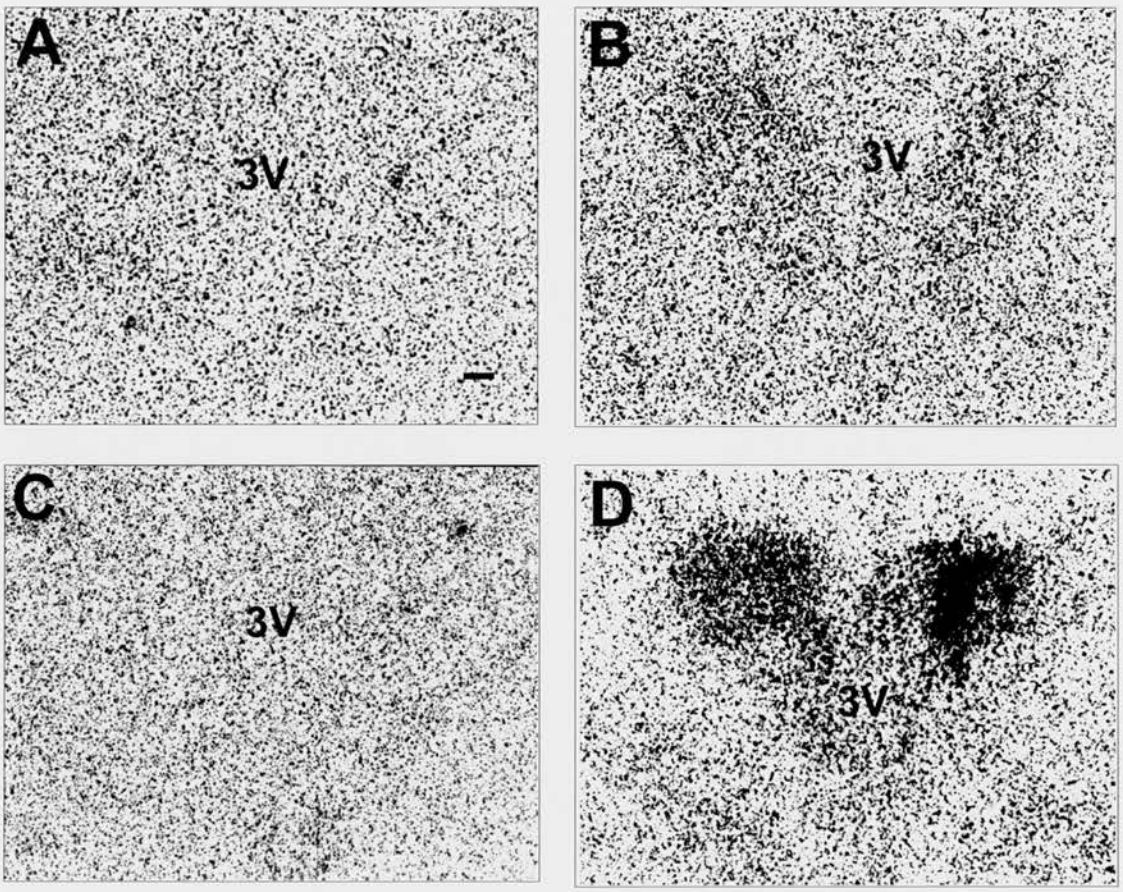
6.3.2. Effect of 48h pharmacological adrenalectomy on basal expression of AVP mRNA in the pPVN

In the vehicle treated rats, levels of AVP mRNA expression in the pPVN tended to be less in the pregnant group compared to the virgin group, however this difference was not statistically significant (figure 6.2.). Treatment with metyrapone over 48h and aminoglutethimide to induce phADX resulted in a significant ($p< 0.05$, two-way ANOVA) increase in pPVN AVP mRNA expression in both the virgin and the pregnant groups (figure 6.2.). The increase in AVP gene transcription evoked by 48 h phADX was significantly greater in the pregnant/phADX group (122 % increase) than in the virgin/phADX group (55 % increase) ($p< 0.01$; two-way ANOVA).



<u>Figure 6.1.(a)</u> Effect of 48h pharmacological adrenalectomy on basal levels of CRH mRNA expression in the pPVN of virgin and pregnant rats.

Virgin and pregnant rats were treated with metyrapone (MET; 100 mg/kg s.c.) or vehicle at 8-hourly intervals over a 48h period. The final MET injection was followed 75 min later by an injection of aminoglutethimide (AG; 200 mg/kg s.c.). One hour after the AG/vehicle injection (on day 21 of pregnancy) rats were killed by decapitation. Coronal brain sections were hybridised with a ^{35}S-labelled oligo-probe complementary to CRH mRNA. Autoradiographs were quantified by computer based image analysis. Values are plotted as group means ± SEM for grain area (mm^2/mm^2). Group numbers: virgin/vehicle, n = 8; pregnant/vehicle, n = 6; virgin/phADX, n = 6; pregnant/phADX, n = 5. Two-way ANOVA followed by Student Newman-Keuls multiple comparison test as used for statistical analysis of the data: *$p < 0.005$ vs vehicle treated groups; #$p < 0.05$ vs all other groups.



<u>Figure 6.1.(b)</u> Effect of 48h pharmacological adrenalectomy on basal levels of CRH mRNA expression in the pPVN of virgin and pregnant rats: Photomicrographs.

Brightfield photomicrographs of coronal sections through the paraventricular nucleus hybridised with a ^{35}S-labelled probe complementary to CRH mRNA from A, virgin/vehicle; B, virgin/phADX; C, pregnant/vehicle; D, pregnant/phADX. 3V, third ventricle. Scale Bar: 100μm.

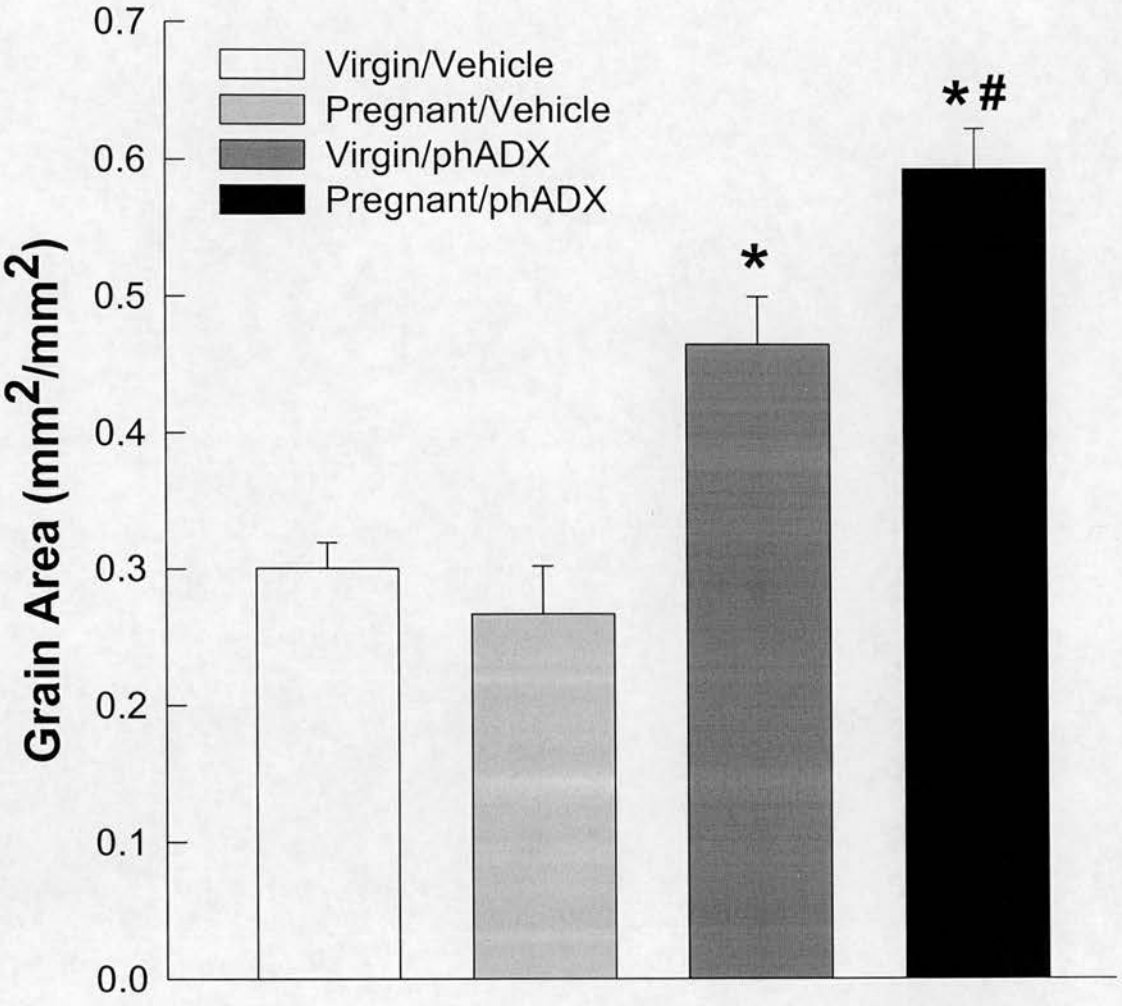


Figure 6.2.(a) Effect of 48h pharmacological adrenalectomy on basal levels of AVP mRNA expression in the pPVN of virgin and pregnant rats.

Virgin and pregnant rats were treated with metyrapone and aminoglutethimide to induce pharmacological adrenalectomy (phADX) as above (see figure 6.1(a) legend). Coronal brain sections were hybridised with a ^{35}S-labelled oligo-probe complementary to AVP mRNA. Autoradiographs were quantified by computer based image analysis. Values are plotted as group means ± SEM for grain area (mm^2/mm^2). For group numbers see figure 6.1(a) legend. Two-way ANOVA followed by Student Newman-Keuls multiple comparison test as used for statistical analysis of the data: *$p < 0.001$ vs vehicle treated groups; #$p < 0.01$ vs all other groups.

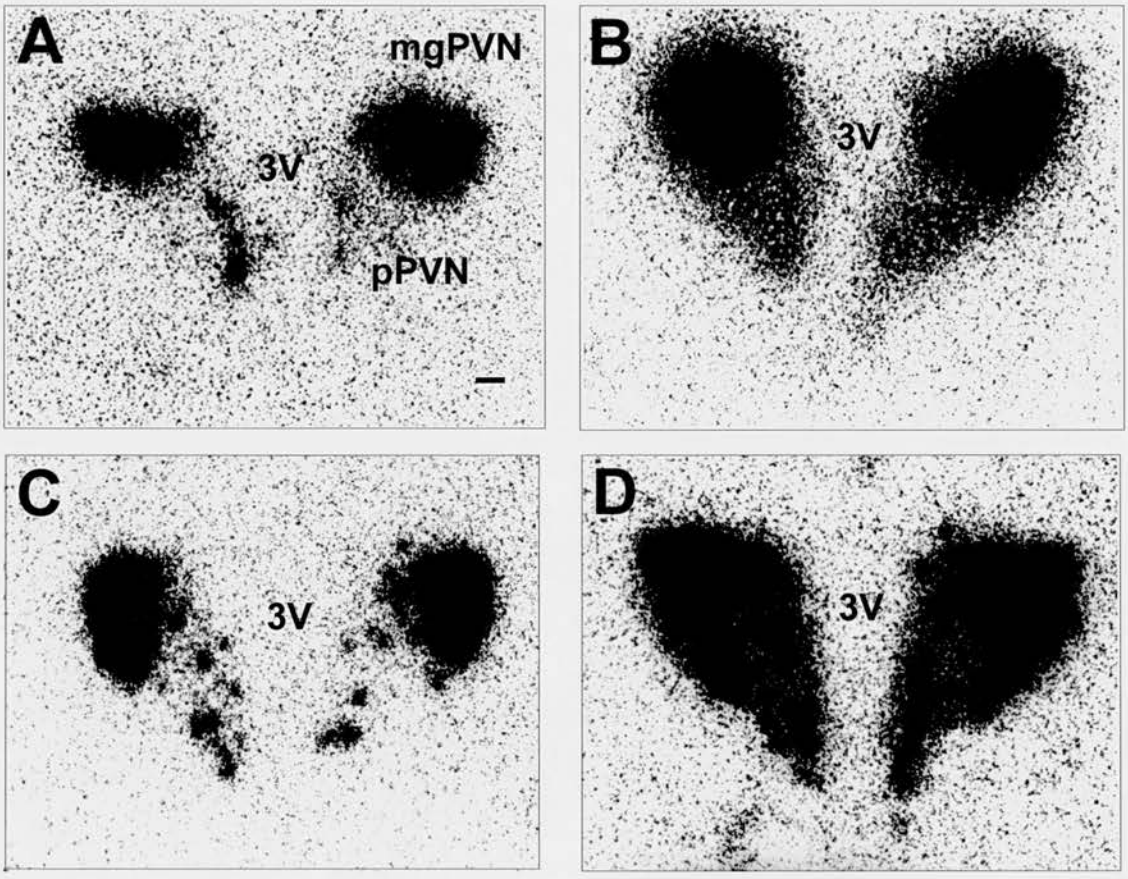


Figure 6.2.(b) Effect of 48h pharmacological adrenalectomy on basal levels of AVP mRNA expression in the pPVN of virgin and pregnant rats: Photomicrographs.

Brightfield photomicrographs of coronal sections through the paraventricular nucleus hybridised with a ^{35}S-labelled probe complementary to arginine vasopressin mRNA from A, virgin/vehicle; B, virgin/phADX; C, pregnant/vehicle; D, pregnant/phADX. 3V, third ventricle; mgPVN, magnocellular division of PVN; pPVN, parvocellular region of PVN. Scale Bar: 100μm.

6.3.3. Effect of 48h pharmacological adrenalectomy on basal plasma ACTH concentrations

Basal concentrations of plasma ACTH were not significantly different between the virgin and pregnant control groups (figure 6.3.). Pharmacological adrenalectomy (over 48 h) evoked a significant increase in plasma ACTH concentration in both the virgin and the pregnant groups, however this increase was significantly greater in the virgin-metyrapone treated group (8.5-fold increase) than in the pregnant metyrapone treated group (4.2-fold increase) ($p< 0.001$; two-way ANOVA).

6.3.4. Effect of 48h pharmacological adrenalectomy on basal plasma corticosterone concentrations

Basal plasma corticosterone concentration in the vehicle treated rats was significantly greater in the pregnant group than in the virgin group (figure 6.4.a). As expected treatment with metyrapone over 48h to induce a state of pharmacological adrenalectomy, caused a significant suppression in corticosterone secretion in both the virgin and the pregnant groups ($p< 0.002$; two-way ANOVA). Although the reduction in plasma corticosterone in the phADX resulted in levels that were not significantly different between the groups, the change in plasma corticosterone concentration was significantly different ($p< 0.01$; Student t-test). There was a 42% reduction in corticosterone secretion in the virgin phADX group compared with a 73% reduction in the pregnant phADX group (figure 6.4.(b)).

6.3.5. Effect of 48h pharmacological adrenalectomy on plasma glucose concentrations

Plasma glucose concentration was significantly lower in the pregnant vehicle treated rats compared with the virgin vehicle treated rats (figure 6.5.). Pharmacological adrenalectomy did not have a significant effect on plasma glucose levels in the virgin group, however phADX caused a significant reduction in plasma glucose concentration in the pregnant group ($p< 0.05$; Kruskal-Wallis, followed by Dunn's post-hoc test).

Treatment with metyrapone or vehicle for 48h had no significant effect on body weight in the virgin rats. As expected, there was a significant increase in body weight (increase of 9.0 ± 2.7 g) in the vehicle treated pregnant rats (after 48h treatment; Students t-test, $p < 0.01$; data not shown). Metyrapone treatment (over 48h), tended to cause a reduction in body weight in the pregnant rats, though this was not significant. A comparison of body weight between the pregnant vehicle treated rats and the pregnant metyrapone treated rats revealed body weight in the latter was significantly reduced (Students t-test, $p < 0.01$; data not shown). This was a consequence of an increase in body weight in the vehicle treated pregnant group and a reduction in body weight in the metyrapone treated group.

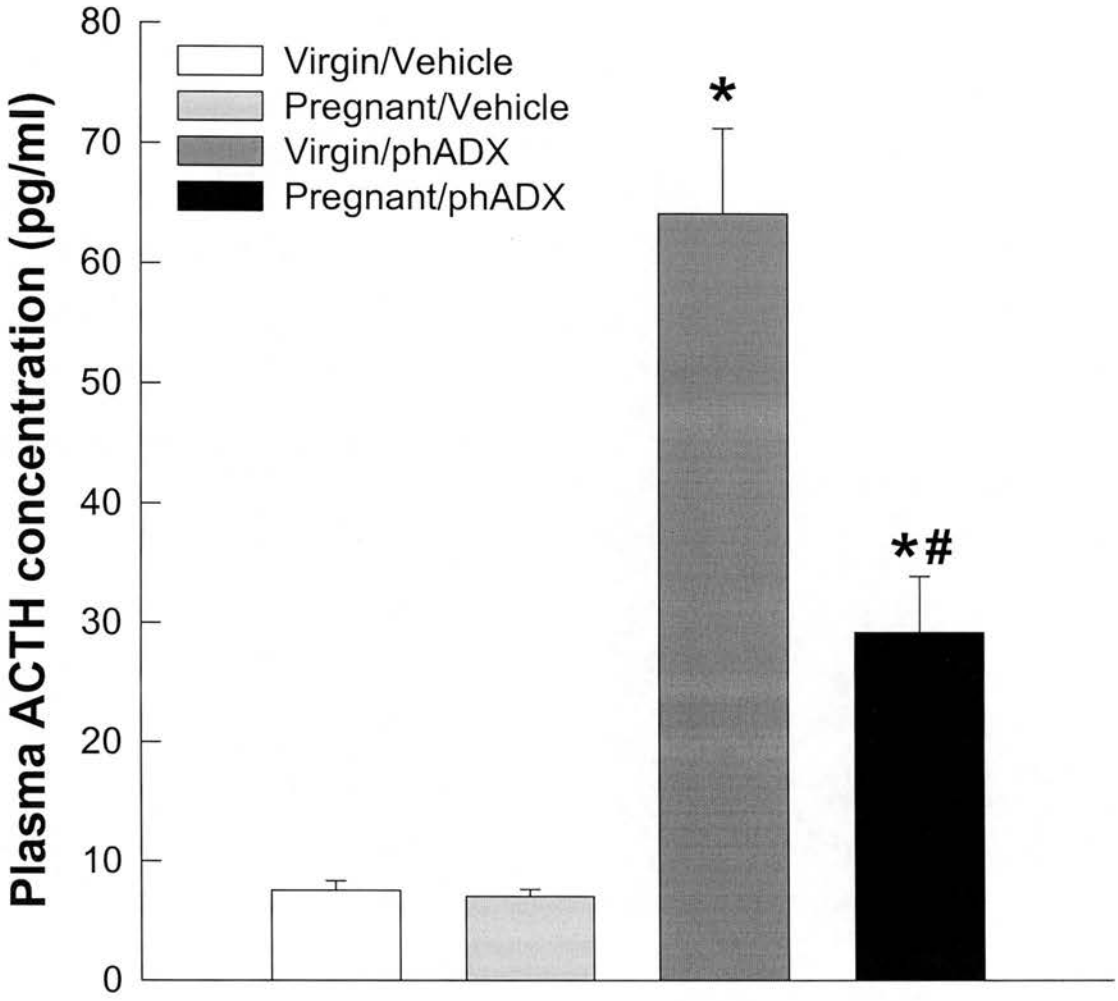


Figure 6.3. Effect of 48h pharmacological adrenalectomy on basal plasma ACTH concentrations in virgin and pregnant rats.

Trunk blood was collected following 48h pharmacological adrenalectomy. Values are plotted as group means ± SEM. For group numbers see figure 6.1(a) legend. Two-way ANOVA followed by Student Newman-Keuls multiple comparison test was used for statistical analysis of the data: *$p < 0.001$ vs vehicle treated groups; #$p < 0.001$ vs all other groups.

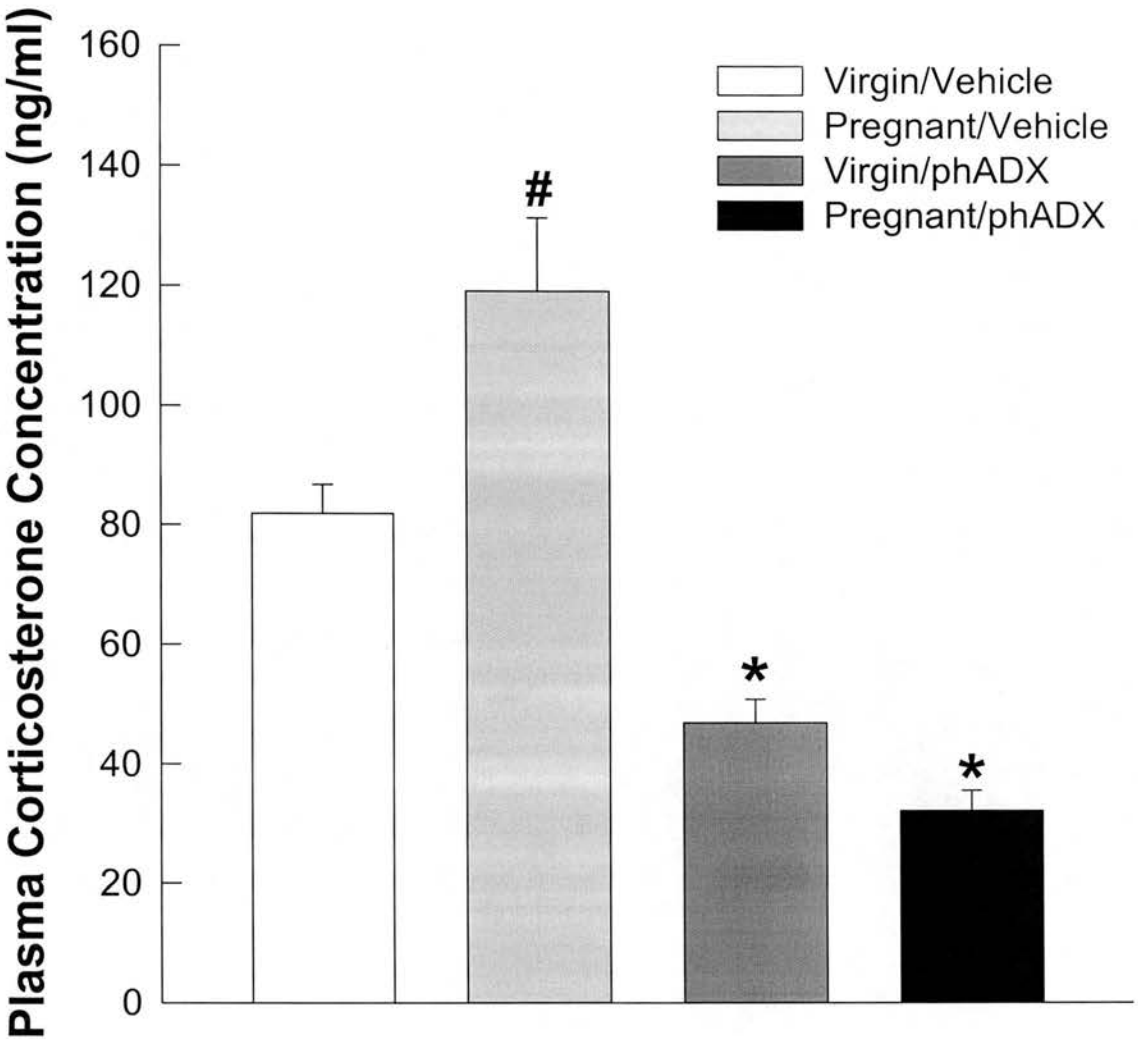


Figure 6.4.(a) Effect of 48h pharmacological adrenalectomy on basal plasma corticosterone concentrations in virgin and pregnant rats.

Trunk blood was collected following 48h pharmacological adrenalectomy. Values are plotted as group means ± SEM. For group numbers see figure 6.1(a) legend. Two-way ANOVA followed by Student Newman-Keuls multiple comparison test was used for statistical analysis of the data: *$p < 0.002$ vs vehicle treated groups; #$p < 0.001$ vs all other groups.

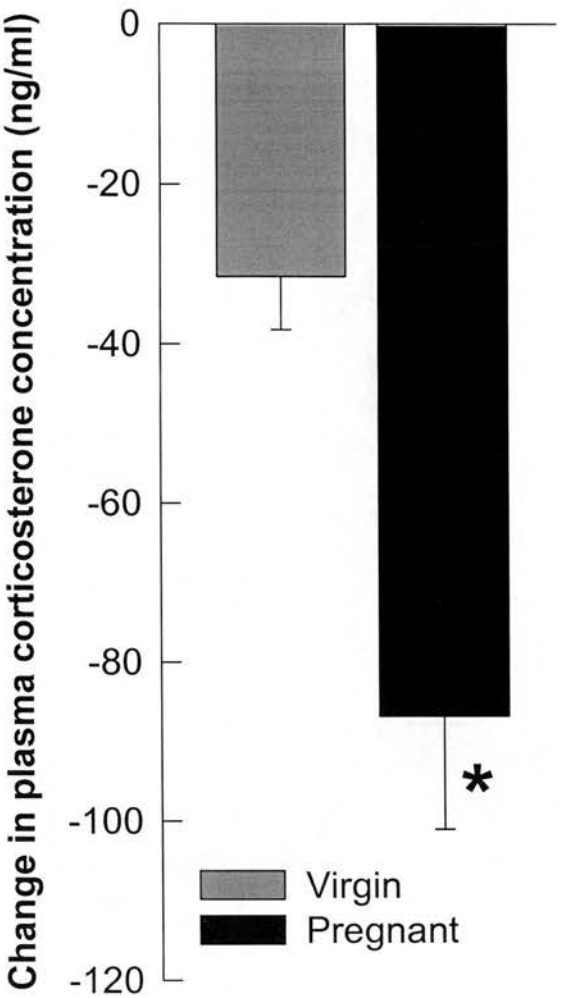


Figure 6.4.(b) Change in plasma corticosterone concentration after 48h pharmacological adrenalectomy in virgin and pregnant rats.

Trunk blood was collected following 48h pharmacological adrenalectomy. Values plotted are change in plasma corticosterone concentration (from their vehicle counterparts) after 48h phADX and are plotted as group means ± SEM. Group numbers: virgin, n = 6; pregnant, n = 6. Students t-test was used for statistical analysis of the data: *$p < 0.01$ vs virgin group.

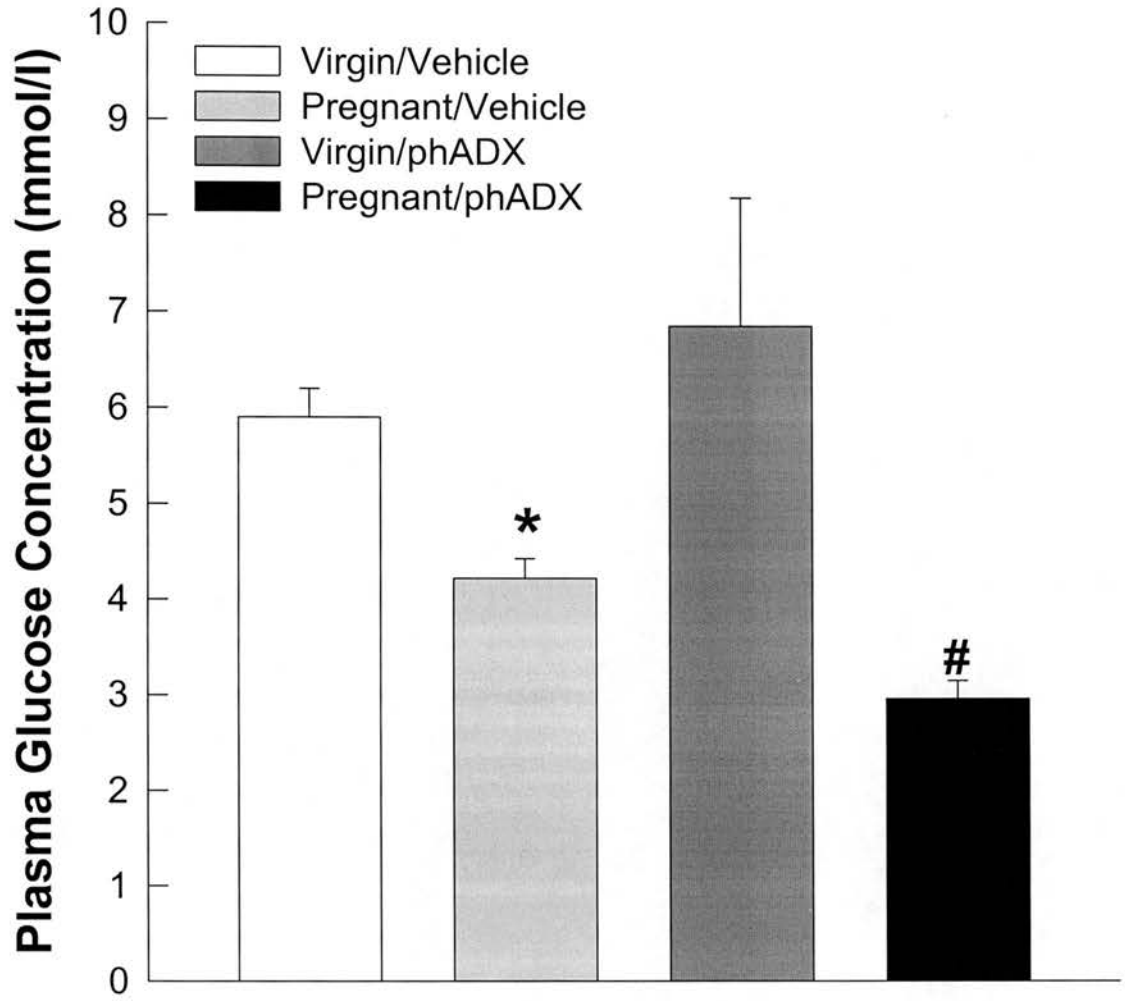


Figure 6.5. Effect of 48h pharmacological adrenalectomy on plasma glucose concentrations in virgin and pregnant rats.

Trunk blood was collected following 48h pharmacological adrenalectomy (see figure 6.1.(a) legend). Values are plotted as group means ± SEM. For group numbers see figure 6.1(a) legend. Kruskal-Wallis followed by Dunn's post-hoc test was used for statistical analysis of the data: *$p < 0.05$ vs virgin/vehicle group; #$p < 0.05$ vs all other groups.

6.3.6. Effect of 48h pharmacological adrenalectomy on adrenal gland weight

Adrenal weight did not differ between virgin and pregnant control groups (but tended to be lower in the pregnant rats; figure 6.6.). In both groups, phADX caused a significant increase in adrenal gland weight relative to the vehicle treated groups ($p< 0.005$; two-way ANOVA), however the increase in the weight of the adrenal glands was greater in the pregnant/phADX (131% increase) group than in the virgin/phADX (45% increase) group.

6.3.7. Effect of 48h pharmacological adrenalectomy on thymus gland weight

Thymus gland weight was significantly lower in the pregnant control rats compared with the virgin control rats (figure 6.7.(a)). Removal of glucocorticoid feedback by phADX did not significantly affect the weight of the thymus gland in the virgin group, however phADX significantly increased thymus gland weight in the pregnant group ($p< 0.02$; two-way ANOVA). Thymus weight was indirectly proportional to plasma corticosterone concentration (figure 6.7.(b)). There was a negative correlation between thymus weight and plasma corticosterone concentration in the pregnant groups, but not in the virgin groups, when the data were analysed separately (figure 6.7.(c)).

6.3.8. Effect of 48h pharmacological adrenalectomy on foetal weight

The combined foetal and placental weight was significantly ($p< 0.02$; two-tailed unpaired t-test) lower in the pregnant/phADX group compared with the pregnant vehicle treated group (figure 6.8.). Further analysis revealed that this reduction was due to a significant reduction in foetal weight ($p< 0.04$) with no significant change in placental weight (data not shown).

The phADX treated rats were not alert and were non-responsive when handled. The reasons for this are not known, but appear to be an unexpected result of phADX. Despite this and the reduction in foetal weight, the foetuses were still alive *in utero* at the end of the experiment. Pregnant rats with dead and/or under-developed foetuses were excluded from the analysis.

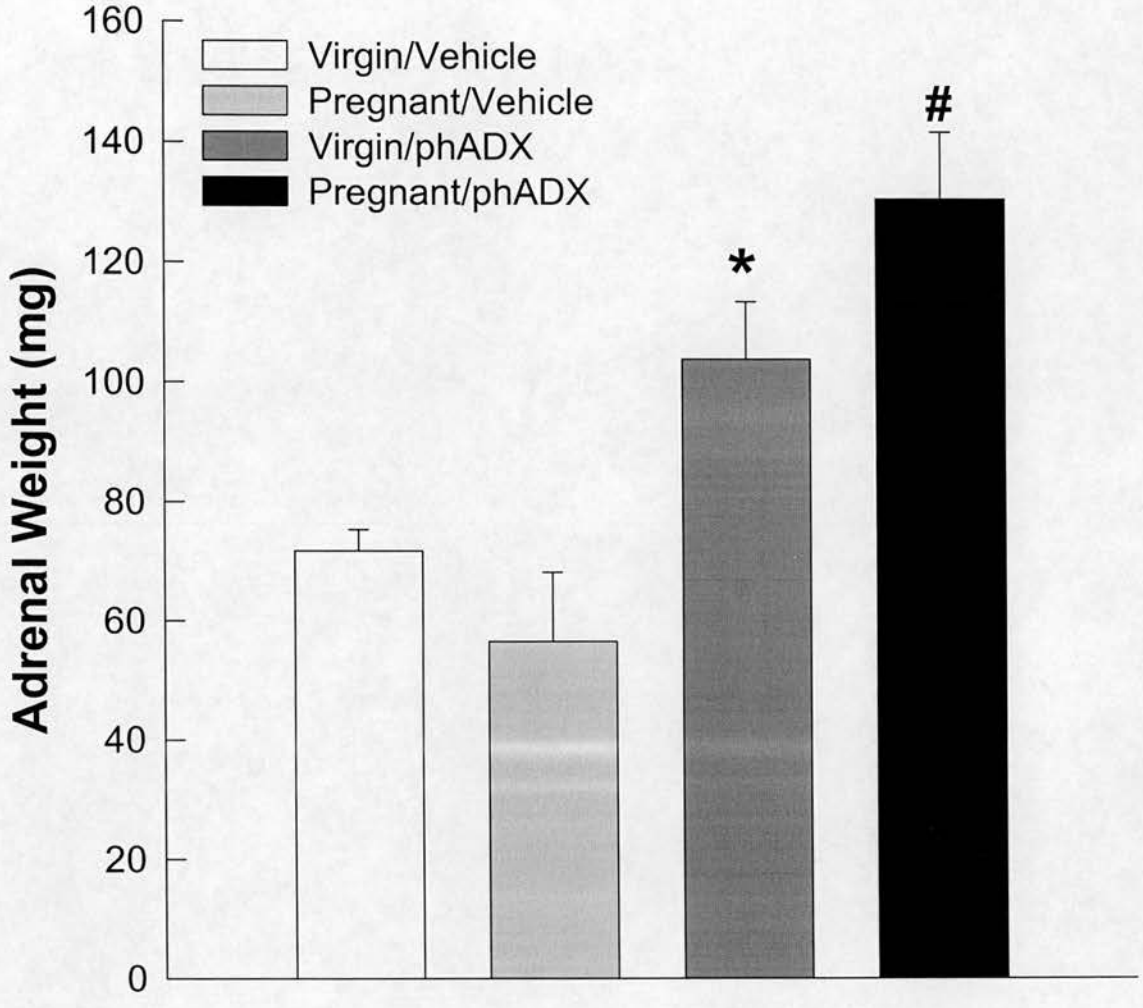


Figure 6.6. Effect of 48h pharmacological adrenalectomy on adrenal gland weights in virgin and pregnant rats.

Rats were killed after 48h pharmacological adrenalectomy (see figure 6.1.(a) legend). Both adrenal glands were dissected from the rat and adipose/connective tissue was removed. The tissue was transferred to eppendorf tubes and weighed. Values are plotted as group means ± SEM. For group numbers see figure 6.1(a) legend. Two-way ANOVA followed by Student Newman-Keuls multiple comparison test was used for statistical analysis of the data: *$p < 0.005$ vs vehicle treated groups; #$p < 0.03$ vs all other groups.

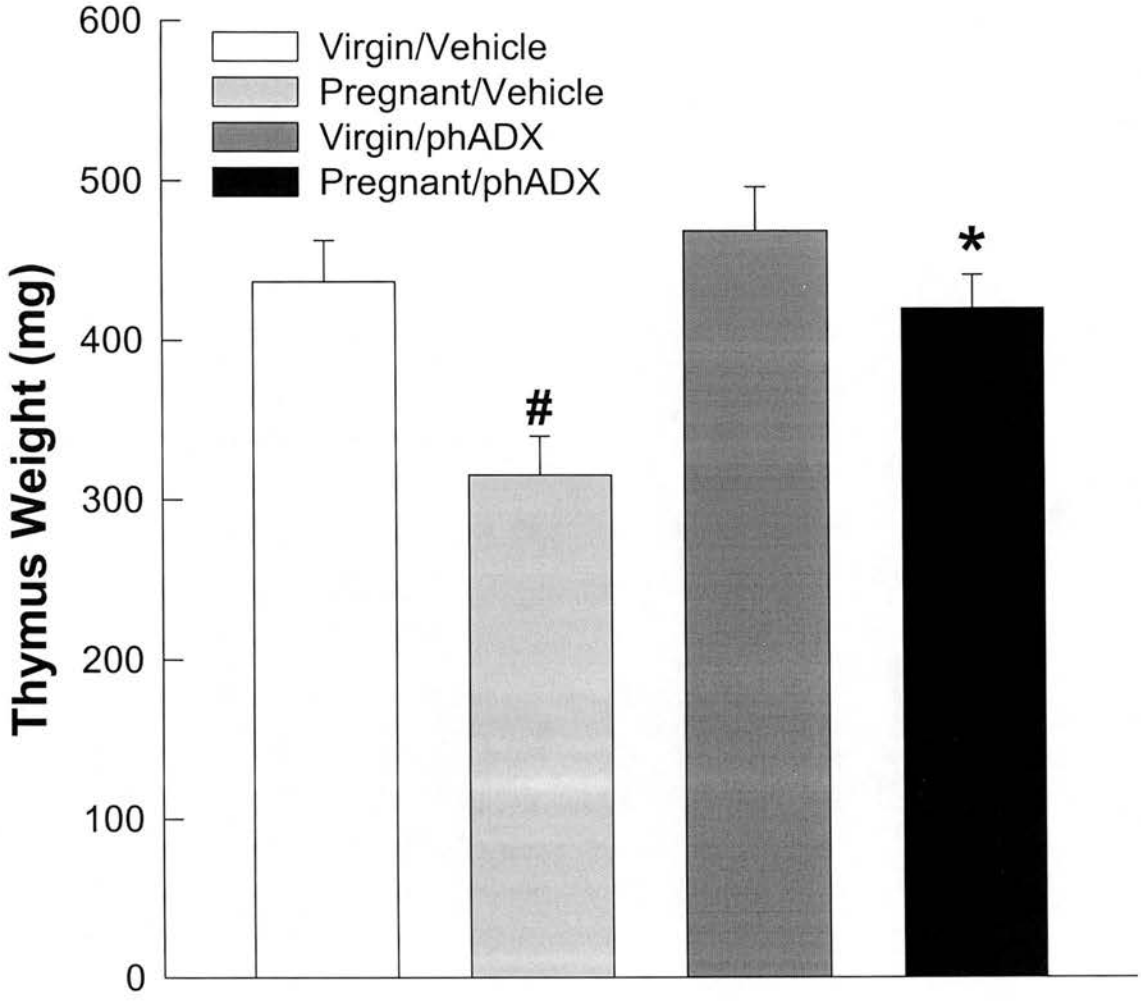


Figure 6.7.(a) Effect of 48h pharmacological adrenalectomy on thymus gland weights in virgin and pregnant rats.

Rats were killed after 48h pharmacological adrenalectomy (see figure 6.1.(a) legend). The thymus gland was dissected from the rat and adipose/connective tissue was removed. The tissue was transferred to eppendorf tubes and weighed. Values are plotted as group means ± SEM. For group numbers see figure 6.1(a) legend. Two-way ANOVA followed by Student Newman-Keuls multiple comparison test was used for statistical analysis of the data: *$p < 0.02$ vs vehicle treated pregnant group; #$p < 0.002$ vs virgin/vehicle group.

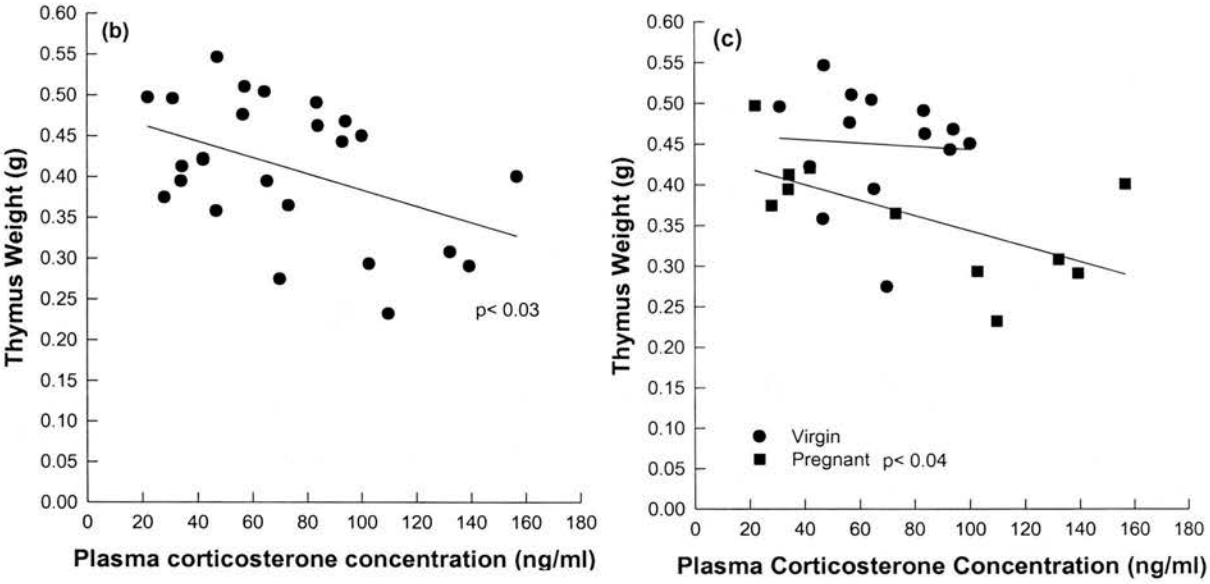


Figure 6.7.(b, c): Relationship between thymus gland weight and plasma corticosterone concentration.

Rats were killed after 48h pharmacological adrenalectomy (see figure 6.1.(a) legend). Trunk blood was collected and subsequently assayed for corticosterone. The thymus gland was dissected from the rat and adipose/connective tissue was removed. The tissue was transferred to eppendorf tubes and weighed.

(b) Values from all rats are plotted as thymus gland weight against plasma corticosterone concentration. There is a significant correlation between thymus gland weight and plasma corticosterone concentration ($p < 0.03$; Pearson Product Moment Correlation test; the correlation coefficient is -0.4347).

(c) Values from the virgin and the pregnant rats are plotted separately as thymus weight against plasma corticosterone concentration. There is a significant correlation between thymus gland weight and plasma corticosterone concentration in the pregnant group ($p < 0.04$; Pearson Product Moment Correlation test; the correlation coefficient is -0.6339), but not in the virgin group.

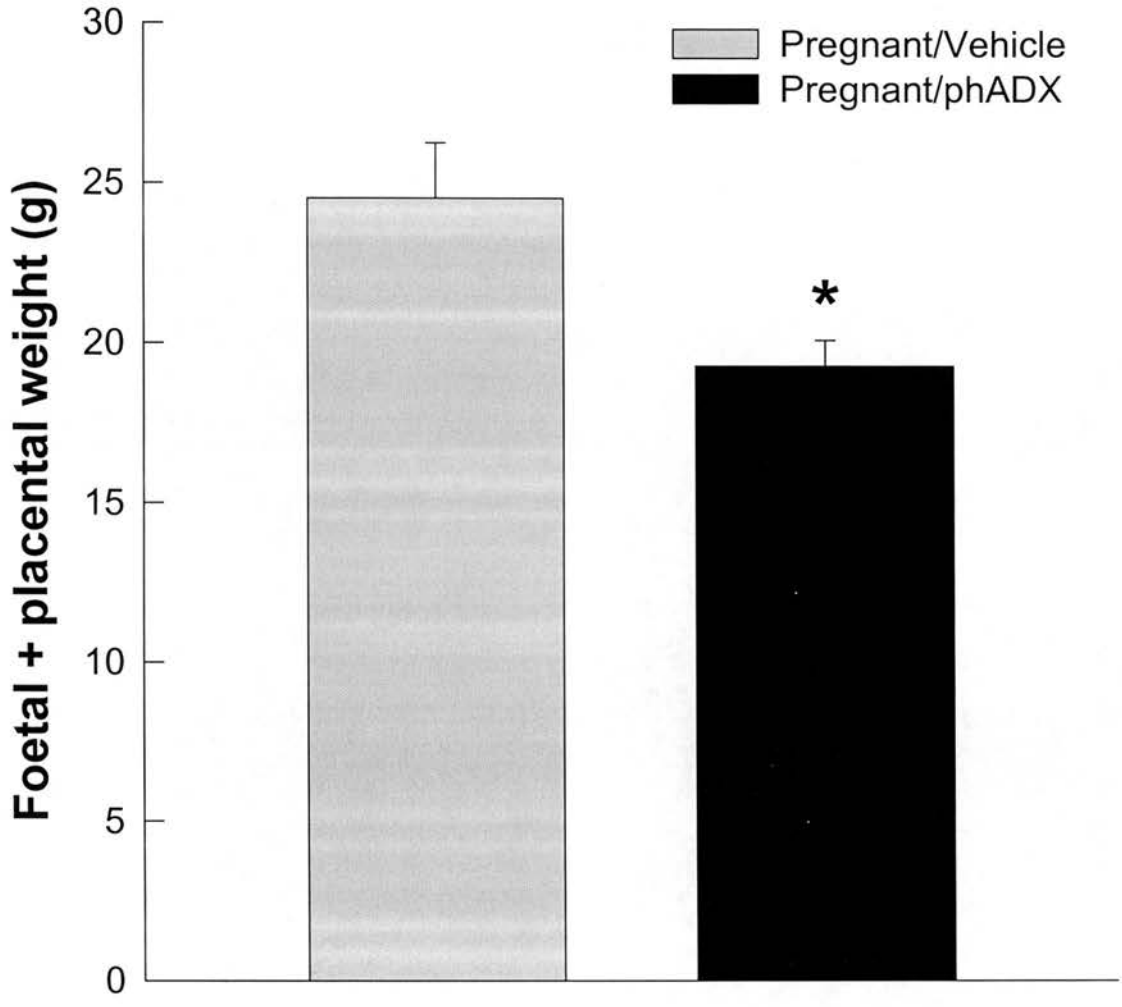


Figure 6.8. Effect of 48h pharmacological adrenalectomy on foetal weights in pregnant rats.

Rats were killed after 48h pharmacological adrenalectomy (see figure 6.1.(a) legend). Five pups (with placentae attached) were removed from the pregnant rat uteri and weighed. Values plotted are group means ± SEM. Group numbers: pregnant/vehicle, n = 6; pregnant/phADX, n = 5. Two-tailed unpaired t-test was used for statistical analysis of the data: *$p < 0.02$ vs vehicle treated group.

Experiment 2: Effect of 24h pharmacological adrenalectomy on stress-induced HPA activity

6.3.9. Effect of 24h pharmacological adrenalectomy on stress-induced plasma ACTH concentrations

Prior to any treatment, basal plasma concentrations of ACTH did not differ between any of the groups (figure 6.9.). Subcutaneous vehicle injections over a 24h period had no significant effect on plasma ACTH concentrations in either the virgin or the pregnant group (figure 6.9.). Metyrapone treatment (for 24h) resulted in a significant increase in plasma ACTH levels in both groups ($p< 0.002$; two-way RM ANOVA).

As expected, exposure to 30 min of restraint stress evoked a significant increase in ACTH secretion in the virgin vehicle treated group, which peaked between 15-30 min after the onset of the stress (figure 6.9.). There was also a significant increase in plasma ACTH levels in the pregnant/vehicle group within 15 min of the onset of restraint stress, however this response was significantly attenuated when compared with the virgin/vehicle group ($p< 0.001$; two-way RM ANOVA). Surprisingly, restraint had no effect on plasma ACTH concentration in either the virgin or pregnant phADX groups with plasma ACTH levels not significantly different from basal levels measured after 24 h metyrapone treatment (figure 6.9.). However the phADX treated rats were markedly subdued and non-responsive when handled.

6.3.10. Effect of 24h pharmacological adrenalectomy on stress-induced plasma corticosterone concentrations

Basal levels of corticosterone measured prior to metyrapone treatment were not significantly different between any of the groups, although plasma corticosterone concentration tended to be higher in the pregnant groups (figure 6.10). Following 24h of treatment with metyrapone and a single injection of aminoglutethimide basal levels of corticosterone were measured.

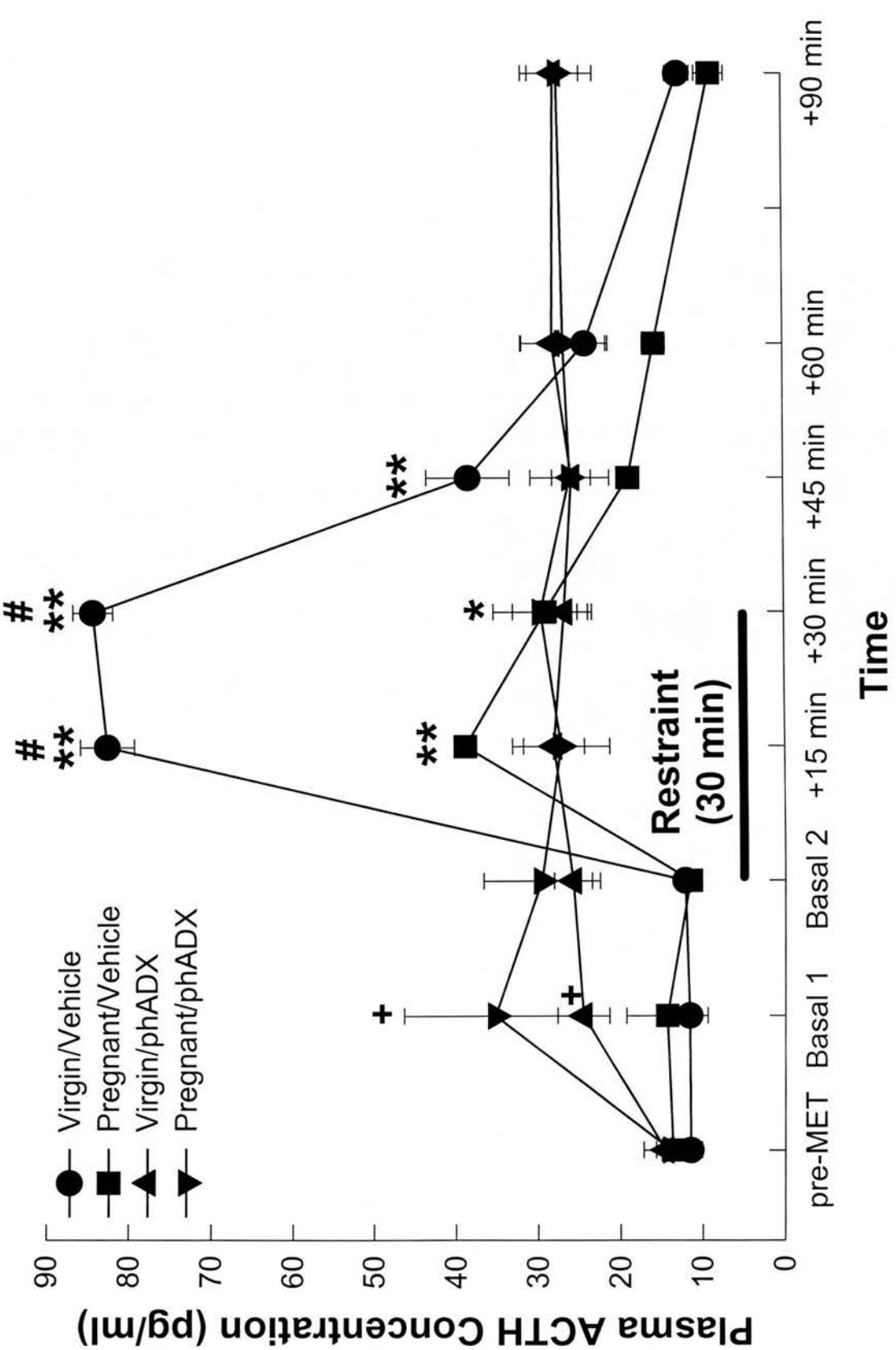

Plasma ACTH Concentration (pg/ml)
0
10
20
30
40
50
60
70
80
90
Virgin/Vehicle
Pregnant/Vehicle
Virgin/phADX
Pregnant/phADX
pre-MET
Basal 1
Basal 2
+15 min
+30 min
+45 min
+60 min
+90 min
Time
Restraint
(30 min)
+
+
**
*
**
#
**
#
**

Figure 6.9. The effect of 24h phADX on plasma ACTH responses to restraint stress in virgin and pregnant rats.

One blood sample was withdrawn prior to any metyrapone treatment (day 20). On the morning of the experiment (day 21), two basal blood samples were collected 30 minutes apart, after 24h metyrapone (4 x 100 mg/kg s.c.) treatment and a single injection of aminoglutethimide (200 mg/kg s.c.). Rats were then exposed to 30 min of restraint stress in Perspex tubes. Further blood samples were withdrawn 15, 30, 45, 60 and 120 minutes after the onset of the period of restraint. Values are plotted as group means ± SEM. Group numbers: virgin/vehicle, n = 5; pregnant/vehicle, n = 5; virgin/phADX, n = 8; pregnant/phADX, n = 7. Two-way ANOVA for repeated measures followed by Student Newman-Keuls multiple comparison test was used for statistical analysis of the data: +$p < 0.002$ vs pre-MET sample; *$p < 0.02$, **$p < 0.001$ vs basal values in the same group; #$p < 0.001$ vs all other groups at the same time point.

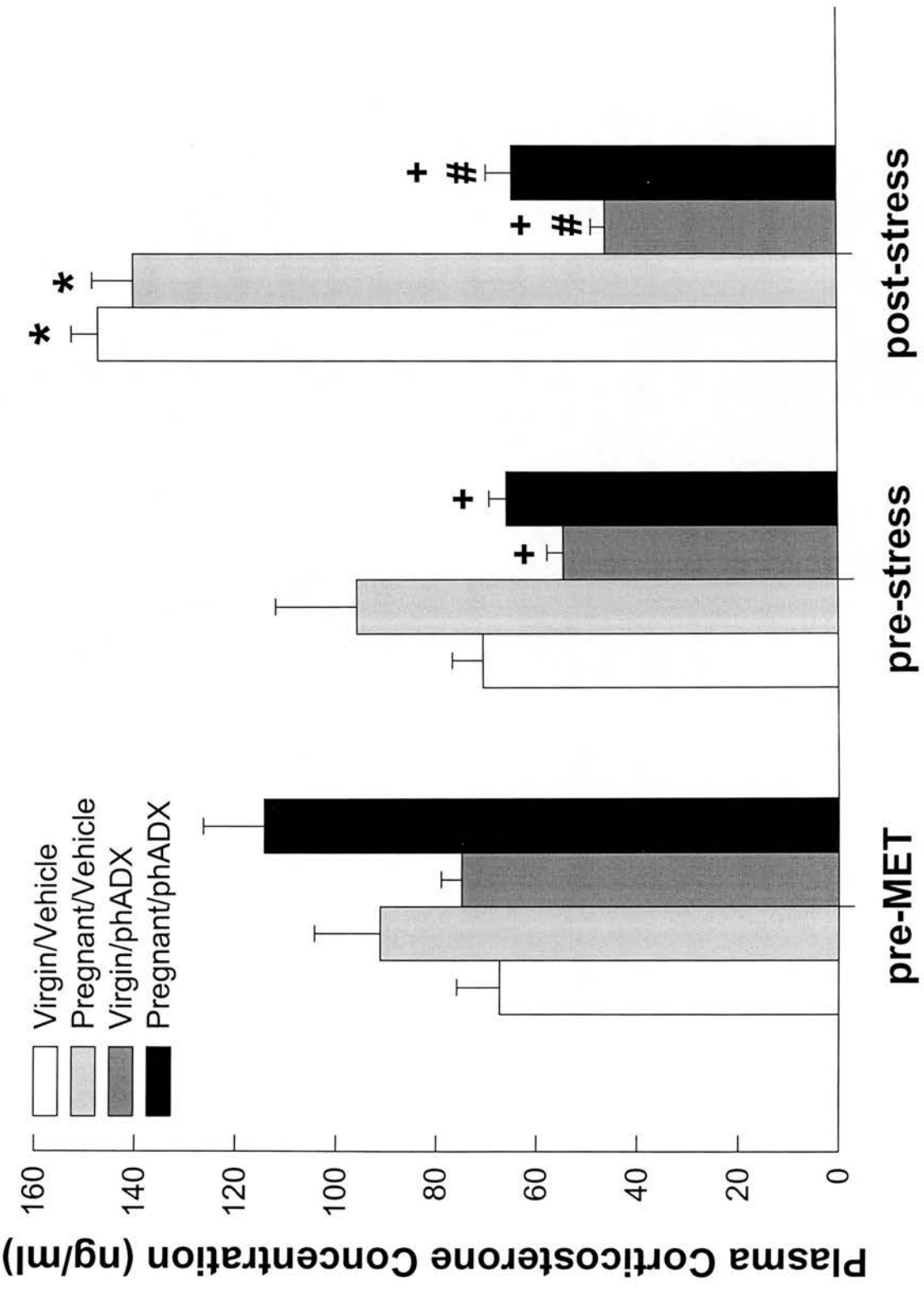

Plasma Corticosterone Concentration (ng/ml)
0
20
40
60
80
100
120
140
160
Virgin/Vehicle
Pregnant/Vehicle
Virgin/phADX
Pregnant/phADX
pre-MET
pre-stress
post-stress
*
*
+
+
+
+
#
#

Figure 6.10. The effect of 24h phADX on plasma corticosterone responses to restraint stress in virgin and pregnant rats.

One blood sample was withdrawn prior to any metyrapone (pre-MET) treatment (day 20) (For treatments see figure 6.9. legend). On the morning of the experiment (day 21), a basal blood sample was collected 90 minutes after aminoglutethimide (200 mg/kg s.c.) administration (pre-stress). Rats were then exposed to 30 min of restraint stress in Perspex tubes. A further blood sample was withdrawn 45 minutes after the onset of the period of restraint (post-stress). Values are plotted as group means ± SEM. For group numbers see figure 6.9. legend. Two-way ANOVA for repeated measures followed by Student Newman-Keuls multiple comparison test was used for statistical analysis of the data: $+p < 0.01$ vs pre-MET sample; $*p < 0.002$ vs pre-MET and pre-stress values in the same group; $\#p < 0.001$ vs vehicle group at the same time point.

Pharmacological ADX over 24h resulted in a significant reduction in plasma corticosterone levels in both virgin and pregnant rats compared with the vehicle injected controls ($p< 0.01$; two-way RM ANOVA), with no difference between the virgin and pregnant phADX groups (figure 6.10). Exposure to 30 min restraint stress caused a significant increase in plasma corticosterone concentration in both the virgin and pregnant vehicle treated rats ($p< 0.002$; two-way RM ANOVA), however this failed to evoke any response in either the virgin or pregnant phADX groups (figure 6.10.).

6.3.11. Effect of 24h pharmacological adrenalectomy on plasma glucose concentrations

Plasma glucose concentrations were not significantly different in either the virgin or pregnant group after 24h phADX compared with their respective controls (figure 6.11.). Plasma glucose levels were lower in both the pregnant groups (pre- and post-treatment) when compared with the virgin/vehicle group (Student t-test; $p< 0.02$).

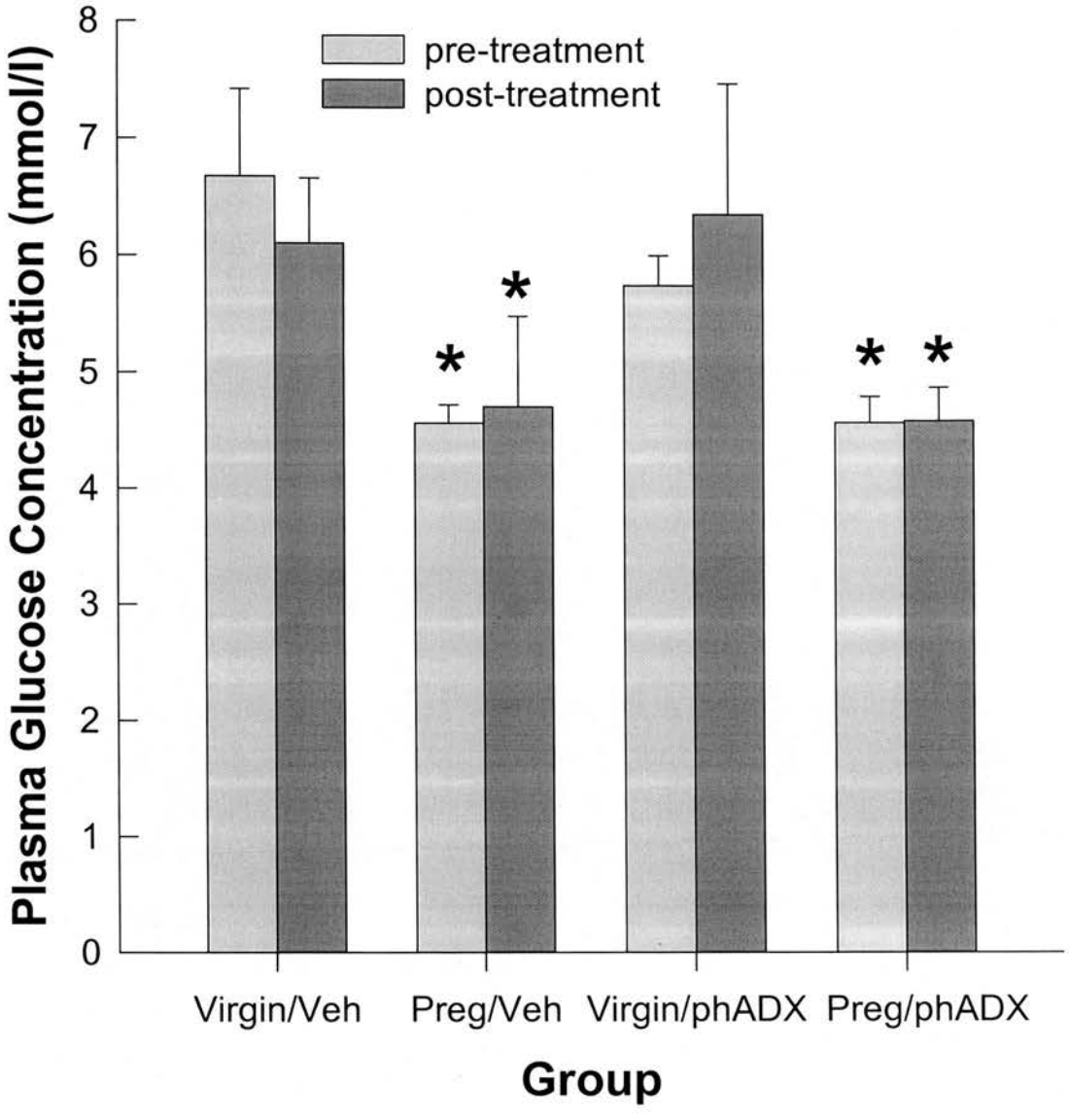


Figure 6.11. The effect of 24h phADX on plasma glucose concentration in virgin and pregnant rats.

Blood samples were withdrawn for measurement of plasma glucose levels before and after treatment with metyrapone (MET; 4 x 100mg/kg s.c.) and aminoglutethimide (200 mg/kg s.c.). Values are plotted as group means ± SEM. For group numbers see figure 6.9. legend. Two-way ANOVA for repeated measures followed by Student Newman-Keuls multiple comparison test indicated no significant differences within any of the groups after treatment with vehicle or metyrapone treatment for 24h ($p > 0.05$). * $p < 0.02$, significantly lower than virgin/vehicle treated groups (Student t-test).

Experiment 3: Effect of enhanced corticosterone negative feedback on stress-induced HPA activity

6.3.12. Effect of corticosterone pre-treatment on stress-induced plasma ACTH concentrations

Plasma ACTH concentrations were not significantly different between any of the groups under basal conditions (figure 6.12.(a)). Neither corticosterone nor vehicle treatment had any effect on plasma ACTH levels measured 60 min after the injection in any of the groups. As expected, exposure to 90 seconds of forced swimming resulted in a rapid increase (within 5 min) in ACTH secretion in the virgin control group, with levels remaining significantly elevated 50 min after the stress ($p < 0.001$; two-way RM ANOVA). Plasma ACTH concentration was also significantly increased in the pregnant control group 15 min after forced swimming (figure 6.12.(b); $p < 0.01$, repeated measures ANOVA on ranks). Swim stress significantly increased ACTH secretion from basal levels in the virgin group pre-treated with corticosterone, however the magnitude of this increase was significantly lower than that observed in the virgin control group (5.8-fold increase in the virgin control group vs. 2.5-fold increase in the virgin corticosterone treated group). Pre-treatment with corticosterone had no further effect on the ACTH secretory response to forced swimming in the pregnant group, with plasma concentrations not significantly different from the pregnant control group (figure 6.12.(a, b)).

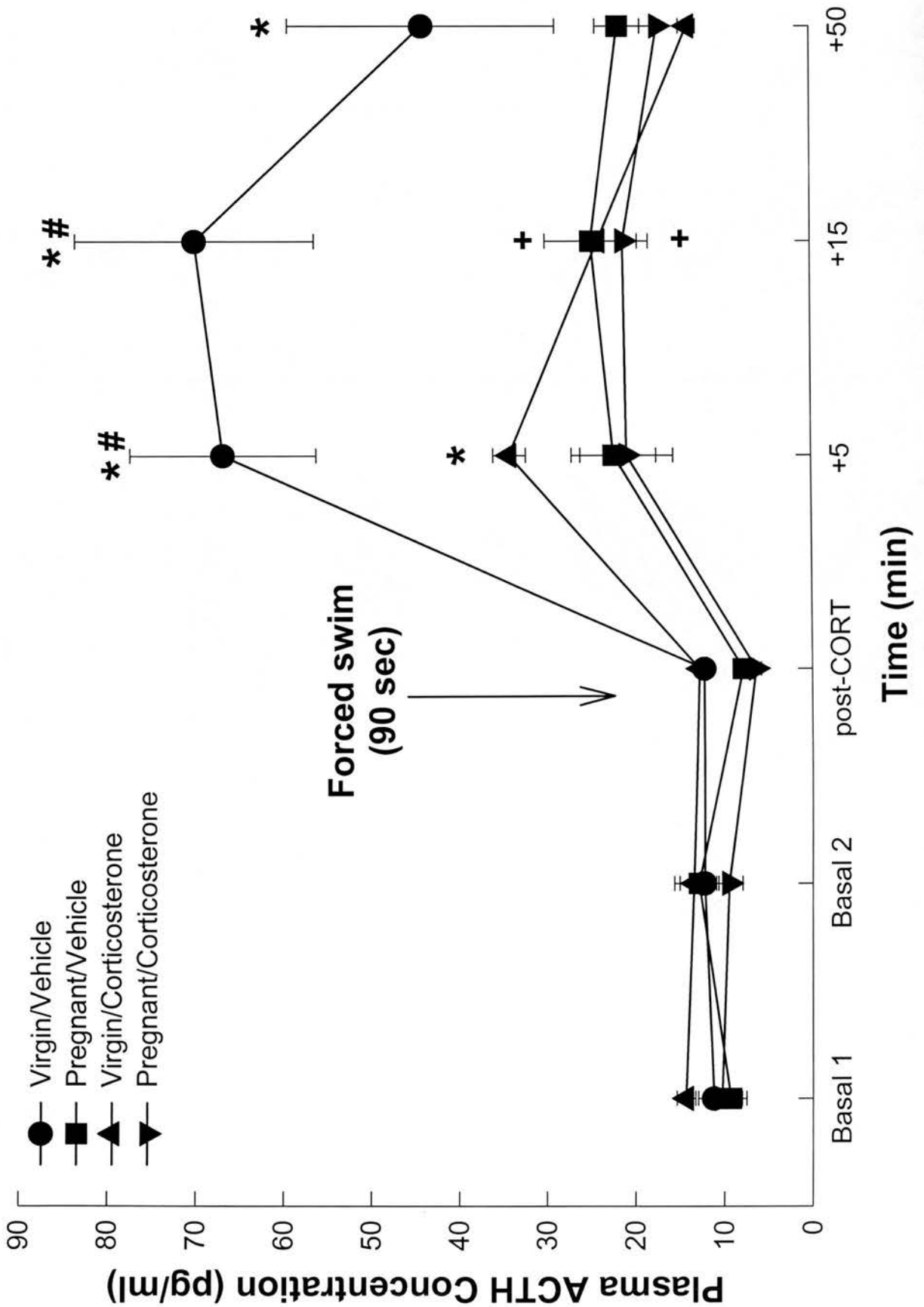

Plasma ACTH Concentration (pg/ml)
0
10
20
30
40
50
60
70
80
90
Time (min)
Basal 1
Basal 2
post-CORT
+5
+15
+50
Forced swim
(90 sec)
Virgin/Vehicle
Pregnant/Vehicle
Virgin/Corticosterone
Pregnant/Corticosterone
*
*#
*#
*
+
+

Figure 6.12.(a) Effect of corticosterone pre-treatment on stress-induced plasma ACTH concentrations

Two basal blood samples were withdrawn 30 min apart. The second blood sample was immediately followed by subcutaneous administration of either corticosterone-21-acetate (2 mg/kg) or vehicle. Sixty minutes after the injection, all of the rats were exposed to 90 seconds forced swimming in water at 19°C. Further blood samples were withdrawn 5, 15 and 50 min after the swim stress. Values are plotted as group means ± SEM. Group numbers: virgin/vehicle, n = 5; pregnant/vehicle, n = 6; virgin/corticosterone, n = 6; pregnant/corticosterone, n = 7. Two-way ANOVA for repeated measures followed by Student Newman-Keuls multiple comparison test was used for statistical analysis of the data: *$p < 0.001$, +$p < 0.01$ vs basal values in the same group; #$p < 0.001$ vs all other groups at the same time point.

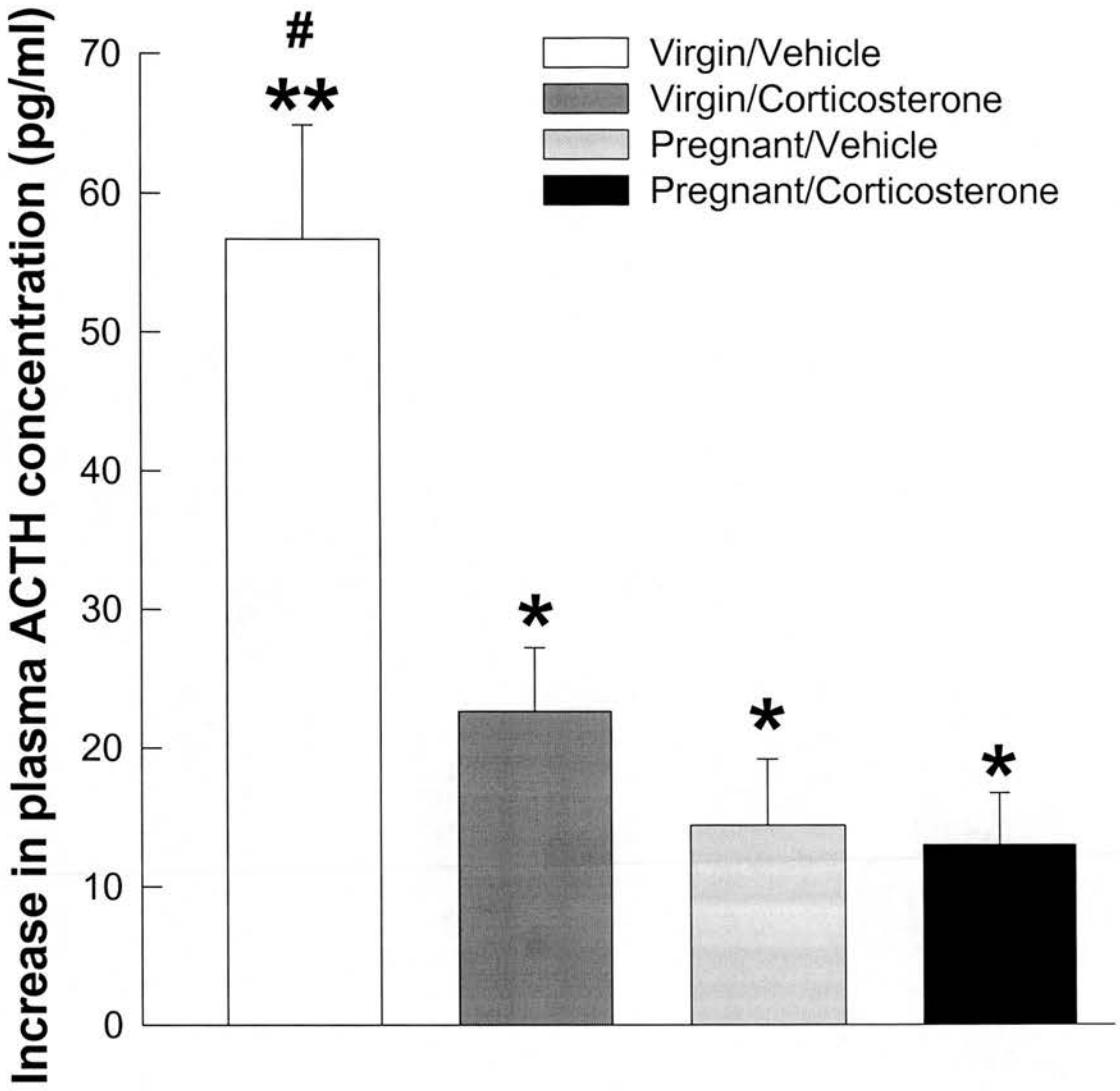


Figure 6.12.(b) Increase in plasma ACTH concentration after corticosterone treatment and forced swimming.

The increase in plasma ACTH concentration at 5 min post-swim (sample 4 on figure 6.12.(a)) from pre-swim levels (sample 3) was calculated and is plotted as group mean ± SEM. Repeated measures ANOVA on ranks followed by Dunn's post-hoc test was used to analyse the data: # indicates the increase in ACTH secretion this group was greater than in any of the other groups ($p < 0.01$). Paired t-test revealed significant increases in plasma ACTH concentration from pre-swim levels (*$p < 0.03$; **$p < 0.005$).

6.4. Discussion

Basal and stress induced activity of the HPA axis is dependent upon integration of all the excitatory and all the inhibitory neural inputs to the pPVN neurones and feedback inhibition mediated via glucocorticoids. In late pregnancy, the balance between stimulation and inhibition shifts, resulting in reduced feed-forward mechanisms (Neumann *et al* , 1998; Johnstone *et al* , 2000a). Here evidence was sought for altered glucocorticoid feedback sensitivity during pregnancy.

Experiment 1: The role of glucocorticoid feedback on basal HPA axis activity in late pregnancy

The present study demonstrated that removal of the glucocorticoid inhibitory feedback signal by phADX resulted in an increase in both CRH and AVP mRNA expression in the parvocellular region of the PVN of both virgin and pregnant rats. These results support a role for tonic glucocorticoid inhibition in regulating CRH and AVP gene expression, which has previously been described following surgical adrenalectomy (Young *et al*, 1986). If it is assumed that the mRNA is translated into protein, then by preventing the feedback actions of corticosterone it follows that phADX increases production of CRH and AVP. In the pregnant group phADX induced a significantly greater increase in PVN CRH and AVP mRNA expression under basal conditions, suggesting that in late pregnancy the PVN neurones are more sensitive to slow glucocorticoid negative feedback. Increased sensitivity to the negative feedback actions of glucocorticoids in pregnancy has previously been suggested from a study demonstrating increased GR mRNA expression in the dentate gyrus in late pregnant rats and increased 11β-HSD activity in the PVN (Johnstone *et al* , 2000a). However this same study reported reduced levels of basal CRH and AVP mRNA expression in the PVN, an effect not observed in the control rats of the present experiment. This is likely to be a result of the stress associated with handling and administration of the vehicle injections.

Under basal conditions, Atkinson and Waddell have reported a decrease in levels of plasma ACTH as pregnancy progresses (Atkinson & Waddell, 1995). Here, no differences in plasma ACTH concentration were observed between the virgin and pregnant control groups. This can be easily explained. The evidence presented by Atkinson and Waddell to support reduced ACTH secretion in late pregnancy, demonstrated reduced mean daily levels in plasma ACTH in pregnancy, however this reduction in ACTH secretion was due to a loss of the evening rise in ACTH levels with morning basal levels not significantly affected by pregnancy. Here plasma ACTH was measured at one time point during the morning at a time when ACTH secretion is at its lowest and there is little variation between virgin and pregnant rats.

Pharmacological ADX evoked a significant increase in ACTH secretion in both the virgin and the pregnant groups. This is consistent with the reported effects of surgical ADX on plasma ACTH levels (Plotsky & Sawchenko, 1987). The increase in ACTH secretion observed after phADX is likely to be a consequence of increased drive to the pituitary corticotropes by CRH and/or AVP. Since corticosterone also has negative feedback effects at the level of the anterior pituitary, by blocking these actions it would be expected that ACTH release would be increased. However, surprisingly the increase in ACTH levels following 48h phADX was greater in the virgin (7-fold increase) than in the pregnant group (3-fold increase). This result is difficult to interpret, especially as CRH and AVP mRNA levels were greater in the pregnant group, though seems to suggest a change in the sensitivity of the anterior pituitary to CRH and/or AVP in late pregnancy. There are several possibilities: pituitary corticotropes may fail to up-regulate receptor levels sufficiently, indeed it has previously been reported that CRH binding sites decline in late pregnancy (Neumann *et al* , 1998), which may contribute to the reduced ACTH response after phADX in the pregnant group. Alternatively there may be a depletion of ACTH stores, resulting from reduced production of ACTH from its precursor POMC. Lastly, increased CRH and AVP mRNA expression in the pPVN does not necessarily mean that CRH and AVP secretion (or indeed peptide production) are increased simultaneously. Thus,

although removal of glucocorticoid inhibition increases CRH and AVP mRNA expression, it may still be that reduced forward drive (see next experiment) restrains CRH and/or AVP secretion (and hence ACTH secretion) more in pregnant rats. Following this explanation, feedback restraint of ACTH secretion in late pregnancy is less effective on ACTH secretion than in virgins.

Basal corticosterone levels were found to be significantly greater in the pregnant control group compared with the virgin control group. Others have also reported an increase in circulating corticosterone in late pregnancy (Atkinson & Waddell, 1995; Neumann *et al* , 1998). Several hypotheses have been suggested to explain the increase in corticosterone secretion at this time. As the foetal adrenal glands mature they begin to secrete corticosterone, which is detectable from ~ day 17 of gestation (Dupouy *et al*, 1975; Waddell, 1993), although placental 11β-HSD should limit the amount of corticosterone that crosses from the foetus to the maternal circulation. Increased production of oestrogen in pregnancy is reported to increase the sensitivity of the adrenal gland to ACTH (Carr *et al*, 1981; Burgess & Handa, 1992). Whether these elevated concentrations of plasma corticosterone enhance the negative feedback system at the end of pregnancy is not clear as increased circulating CBG (Waddell & Atkinson, 1994) is likely to limit the feedback effects in late pregnancy.

Following 48h phADX, plasma corticosterone levels were significantly reduced to similar levels in the virgin and pregnant groups, indicating that the treatment was successful, however the reduction in corticosterone secretion was greater in the pregnant group. Thus the different effects of phADX on basal ACTH secretion in virgin and pregnant rats is not attributable to a lesser reduction in corticosterone production in pregnant rats. However, the greater overall percentage decrease in corticosterone secretion in the pregnant rats may explain the greater increase in pPVN CRH and AVP mRNA expression. Other parameters were measured to indicate the success of the phADX, namely thymus and adrenal gland weight. In both virgin and pregnant rats, 48h phADX increased the weight of the adrenal glands. These effects are probably a result of increased ACTH drive which leads to

adrenal hypertrophy (Carr *et al* , 1981). The increase in adrenal gland weight was significantly greater in the pregnant group than in the virgin group. Given that these rats have decreased ACTH drive (compared with the virgin phADX group), the greater increase in adrenal weight indicates that the sensitivity of the adrenal gland to ACTH (or other POMC products) increases in pregnancy.

Glucocorticoids have immuno-suppressive actions and act on the thymus gland to inhibit inappropriate responses (Brown, 1998). Thymus weights were significantly lower in the pregnant control group compared with the virgin control group, perhaps a consequence of the elevated corticosterone levels in pregnancy. Pharmacological ADX had no significant effect on thymus weight in the virgin group, however it significantly increased thymus weight in the pregnant group. These data suggest that in pregnancy the thymus gland is more sensitive to the effects of corticosterone.

A primary role of glucocorticoids is to mobilise glucose from glycogen stores. The present results indicate increased metabolic demand during late pregnancy, reflected by significantly lower plasma glucose levels in the control pregnant rats than in the virgins. This may represent the increasing energy demand made by the fetuses, despite increased maternal food intake (Johnstone & Higuchi, 2001). Treatment with metyrapone did not have any significant effect on plasma glucose concentrations in the virgin group, however phADX further decreased glucose levels in the pregnant group. The decreased plasma glucose concentration in phADX pregnant rats may have potentiated the effects of reduced corticosterone secretion on CRH and AVP mRNA expression in the pPVN as glucose provides an important signal in the regulation of the HPA axis (Bhatnagar *et al*, 2000; Bell *et al*, 2000; Laugero *et al*, 2001; Laugero, 2001). Glucocorticoids are essential in pregnancy to cope with the increased demand for energy (Waddell & Atkinson, 1994). Indeed this study indicated that foetal weight was significantly lower when mothers were treated over 48h to induced phADX, which is likely to be due to reduced nutrient transfer, as indicated by maternal glucose levels or a lack of

corticosterone actions (e.g. cellular uptake of amino acids and glucose and storage of glycogen by the liver) in the foetuses.

Experiment 2: Effect of 24h pharmacological adrenalectomy on stress-induced HPA activity

In the second experiment, restraint induced a significant increase in ACTH secretion in both the virgin and pregnant vehicle treated groups and this response was significantly attenuated in the pregnant group. These results are consistent with those presented in Chapter 3 where plasma ACTH concentrations were measured in trunk blood following 30 min restraint stress. If enhanced corticosterone negative feedback is responsible for the reduced responsiveness of the HPA axis in pregnancy, it would be expected that removing corticosterone negative feedback by phADX would enhance the ACTH response to stress. However this was not the case. Neither of the phADX groups mounted an ACTH response when exposed to restraint stress, suggesting that the ability to generate a stress response was absent in these rats, presumably as an unexpected result of the phADX process. There are several possible explanations as to why the ACTH response was absent. Rats treated to induce phADX (in both experiments 1 and 2) displayed were lethargic, evidently unaware of their environment and non-responsive to handling. The reasons behind this behaviour are not clear, although plasma glucose levels tended to be lower in the pregnant groups, levels in neither the virgin nor pregnant groups were significantly affected by phADX over 24 hours. Since these experiments were performed, Bell and colleagues (Bell *et al* , 2000) have reported that a sucrose supplement in addition to drinking water counteracts the metabolic deficits of surgical adrenalectomy (provided the rats have previously learned to drink the sucrose solution). Whether this treatment would have restored normal behaviour in our phADX rats is not known, but should be kept in mind when interpreting the results. In hindsight, it may have been more appropriate to use a different stressor such as forced swimming, which would have required a response from the rats or to have stimulated ACTH secretion by administration of IL-1β. It is possible that after 24h phADX, increased drive by CRH and/or AVP resulted in depletion of anterior

pituitary ACTH stores, thus rendering the rats unable to respond to further stimulation. This seems unlikely since plasma ACTH concentrations remained significantly elevated after 48h phADX in experiment 1. It is possible that following prolonged stimulation the ACTH stores become depleted within 24h and that up-regulation of ACTH from POMC takes longer than this (but less than 48h).

The plasma corticosterone data showed that corticosterone production was significantly decreased after 24h in the phADX groups. It is difficult to interpret the corticosterone results after stress. Neither the virgin nor the pregnant group responded to the restraint stress, however whether this was a result of phADX or a result of a lack of ACTH drive is not clear.

Metyrapone blocks the final step in the pathway of corticosterone production, which presumably will cause a build-up of precursors, many of which are sex steroids; and because of increased ACTH secretion their production will be stimulated. Due to their lipophilic nature, steroids can easily cross the blood brain barrier. It is possible that these steroids are "neuroactive" (Rupprecht & Holsboer, 1999) and indeed may be affecting the brain and actively blocking a stress response in these rats. It has previously been shown that neuroactive steroids involved in biosynthesis of corticosterone from cholesterol are capable of binding to the $GABA_A$ receptor subunit and modulating their activity (Truss & Beato, 1993). The steroids, 3α, 5α- tetrahydraprogesterone and 3α, 5α- tetrahydrodeoxy-corticosterone can bind to $GABA_A$ receptors, where they increase the Cl^- current as well as the frequency and duration of openings of the chloride ion channel, thus enhancing the inhibitory effects of GABA. Thus increased levels of neuroactive steroids (as a result of blocking the final step in the biosynthetic pathway of corticosterone) may be actively restraining the HPA axis response to stress following phADX. To further investigate the HPA axis without these complications it would be necessary to employ a specific antagonist to corticosterone. RU 486 is a GR antagonist which has been used in the past to study feedback effects on the HPA axis, however this compound also inhibits progesterone actions which would compromise the pregnancy and thus would be an unsuitable option. A selective GR

antagonist has recently become available and it would be interesting to study its effects on HPA axis responses in pregnancy.

Experiment 3: Effect of enhanced corticosterone negative feedback on stress-induced HPA activity

In the vehicle treated groups exposure to forced swim stress evoked a rapid increase in ACTH secretion in virgin rats, an effect which was significantly attenuated in the pregnant/vehicle treated group. These results are consistent with previous studies employing the same stressor (Douglas *et al*, 1998; Neumann *et al* , 1998). Acute corticosterone administration resulted in a dramatic reduction in stress-induced ACTH secretion in the virgin group, whereas in the pregnant group pretreatment with corticosterone had no significant effect. This insensitivity to acute feedback can be interpreted in two ways. First, the results are consistent with loss of corticosteroid feedback in this time-domain. A previous study on acute corticosterone effects on ACTH secretion in phADX pregnant and virgin rats showed reduced sensitivity in phADX pregnant rats (Johnstone *et al* , 2000a). Although peak secretion of ACTH following stress in pregnant rats, is less than in virgin rats, ACTH secretion frequently shows a more prolonged response in pregnant rats compared with virgins (Douglas *et al* , 1998; Neumann *et al* , 1998). This is not compatible with greater rapid feedback. Secondly, the present findings superficially support a role for enhanced negative feedback in late pregnancy. By enhancing the feedback signal with exogenous corticosterone, the ACTH response to forced swimming was attenuated in virgin rats. However corticosterone administration had no further effect on lowering the ACTH response to the same stress in the pregnant group. It could be that in late pregnant rats, the HPA axis is already under maximal corticosterone feedback inhibition, thus the animals demonstrate insensitivity to enhanced rapid corticosterone feedback.

Although basal expression of CRH and AVP mRNA in the pPVN neurones increased more in late pregnancy 48h after phADX than in virgin rats, this may

have been a consequence of the greater decrease in corticosterone secretion in pregnant rats, rather than increased sensitivity to slow feedback mechanisms.

In conclusion, it seems unlikely that enhanced *rapid* negative feedback is involved in the reduced responsiveness of the HPA axis to acute stress in pregnancy and instead, if corticosterone feedback is involved it is a consequence of more prolonged exposure to elevated corticosterone levels associated with slow-feedback mechanisms, reducing the capacity of CRH/AVP neurones to respond.

CHAPTER 7

The responsiveness of the hypothalamo-pituitary-adrenal axis to stress during pregnancy in mice

7.1. Introduction

Non pregnant mice, like rats, display robust activation of the HPA axis in response to a range of emotional (e.g. restraint and immobilisation) (Chesnokova *et al*, 1998; Dunn & Swiergiel, 1999) and physical (e.g. LPS and IL-1β) (Delrue-Perollet *et al*, 1995; Neveu & Liege, 2000) stressors. To date no study has investigated the responsiveness of the HPA axis to stress during pregnancy in mice. Here a comparative study was performed to determine whether the HPA axis is hyporesponsive during pregnancy in other species as it is in rats. The model used to test this was the mouse.

The use of transgenic animals as a tool to research the HPA axis has dramatically increased over the last few years. Transgenic animals have been generated with defined defects or 'knockout' of genes with roles in various components of the HPA axis and autonomic nervous system (eg. CRH (Muglia *et al*, 1996; Dunn & Swiergiel, 1999; Venihaki & Majzoub, 1999; Muglia *et al*, 2000), CRH-R (Smith *et al*, 1998), 11β-HSD (Holmes *et al*, 2001) and GR (Dijkstra *et al*, 1998) knock out mice). Although these models provide valuable and novel insights into the development, function, interactions and organisation of the stress system, they have one major drawback, that is, that the vast majority of transgenic models have been developed in mice. Physiology of the HPA axis in mice has been much less studied than this system in the rat. Thus in order to take advantage of this 'new' tool to study the HPA axis in pregnancy, it is important that first the responsiveness of the HPA axis to stress in normal wildtype mice is evaluated in pregnancy.

In the present set of experiments virgin and pregnant mice were exposed to two different stressors: confinement to a glass jar for 10 min (emotional stressor) or 10 min forced swimming at 17°C (combined emotional and physical stressor). Due to the difficulties in cannulating a vein for blood sampling in mice (due to their size) and the limited amount of blood that could be taken for radioimmunoassay, they were killed by decapitation immediately after exposure to the stressor and trunk

blood was collected to determine neuroendocrine stress responses (i.e. ACTH and corticosterone concentrations). To investigate the effects of stress on the hypothalamic pPVN neurones the brains were removed and *in situ* hybridisation was performed for the immediate early gene product, *nur77* mRNA.

Nur77 (also known as NGFI-B in rats) is a member of the steroid nuclear receptor superfamily of transcription factors and it can bind as a monomer to the *cis*-acting sequence, AAAGGTCA, to regulate gene expression, without a requirement for ligand binding (Wilson *et al*, 1991; Davis *et al*, 1991). *Nur77* expression is rapidly induced by neuronal membrane depolarisation (Law *et al*, 1992), thus it provides a good indicator of neuronal activation and it has previously been used to determine the brain areas activated by exposure to stress. *Nur77* mRNA is rapidly induced in the PVN in response to stress (Chan *et al*, 1993; Honkaniemi *et al*, 1994) and central administration of CRH significantly increases expression of *nur77* within the PVN (Parkes *et al*, 1993). *Nur77* transcripts are also strongly induced in the anterior pituitary (the site of POMC synthesis)(Saucedo-Cardenas & Conneely, 1996) and in the adrenal cortex (Davis & Lau, 1994) by stress, as well as in Y1 adrenocortical cells treated with ACTH or cAMP (Wilson *et al*, 1993b; Davis & Lau, 1994). The induction of *nur77* in the adrenal gland has been implicated in the transcriptional regulation of the steroidogenic enzyme, steroid-21α-hydroxylase (Wilson *et al* , 1993b), a rate-limiting enzyme in glucocorticoid synthesis.

Specific DNA-binding sites for *nur77* (NBRE) have been identified in the promoter regions of the CRH and POMC genes (Murphy *et al*, 1995; Murphy & Conneely, 1997), indicating that *nur77* may play a role in regulation of these genes. Indeed *nur77* antagonises the negative feedback effect of glucocorticoids on the synthesis and secretion of ACTH in pituitary corticotropes by binding to the negative glucocorticoid response element in the promoter region of the POMC gene (Okabe *et al*, 1998). Further, glucocorticoids can antagonise the stimulatory effects of *nur77* on CRH and POMC gene transcription (Philips *et al*, 1997; Drouin *et al*, 1998). Thus the *nur77* signalling pathway appears to be a point of convergence for stimulatory signals and glucocorticoid feedback in the neuroendocrine stress system.

The aim of the present experiment was to establish whether HPA axis responses to stressors (confinement to a glass jar and forced swimming) are reduced in pregnancy, and whether as in rats, this involves decreased activation of the pPVN neurones.

7.2. Materials and Methods

7.2.1. Animals

Female BK-white mice (Bantin & Kingman) were used for these experiments. On arrival in the animal unit the mice weighed approximately 30g. From the day of arrival mice were maintained in groups of 6-8 per cage under a reversed 12h light-dark cycle (lights on at 19:00h; the light cycle was reversed to allow studies on parturition (reported elsewhere), which predominantly occurs during the dark phase in mice). Mice were given at least two weeks to acclimatise to this switch in light-dark cycle before mating, and were then maintained with reversed lighting until the end of the experiment. Mice were caged individually from day 14/15 of pregnancy.

7.2.2. Experimental Procedure

The experiment was performed when the pregnant mice were on day 17-18 of pregnancy (the expected day of parturition was day 19) between 19:00-22:00h (i.e. the first 3 hours of the light phase). Mice were exposed to either 10 min forced swimming (in a container 150 mm in diameter; 175 mm in height) in water at 17°C or confinement to an empty glass jar (diameter, 70 mm; height, 120 mm) for 10 min. Immediately after exposure to the stress mice were killed by decapitation. Control mice were left in their home cages until killed by decapitation. Brains were rapidly removed and frozen on dry ice on aluminium foil. Frozen brains were stored at -70°C until processed for *in situ* hybridisation. Trunk blood was collected into Eppendorf tubes on ice containing 100µl 5% EDTA. Plasma was separated by centrifugation and stored at -20°C until hormone assay. [This experiment was performed in collaboration with Drs. A. J. Douglas and I.D. Neumann].

7.2.3. *In situ* hybridisation

Brains were sectioned coronally at 15μm and mounted on slides as previously described in the General Methods Chapter (2). To detect *nur77* mRNA expression a 36-mer oligonucleotide probe was used [MWG-Biotech]. The sequence of the *nur77* mRNA oligo-probe used is given below. It is complementary to bases 201-236 of the mouse *nur77* gene (Hazel *et al*, 1988).

5'- GTC TCG GGG CTG GCC AGG TCC ATG GTA GGC TTG CCG -3'

Probe labelling and hybridisation were performed as previously described (Chapter 2). The post-hybridisation washes were performed in the usual way (Chapter 2). The melting temperature of the *nur77* mRNA oligo-probe is 80°C, therefore the heated SSC washes were performed at 60°C. Once dry, the sections were exposed to autoradiographic film for 28 days at room temperature. The slides were then dipped in autoradiographic emulsion (see Chapter 2) and exposed for 12 weeks. *Nur77* mRNA expression in the pPVN was quantified from emulsion dipped sections using a computer based image analysis system as described in the General Methods Chapter. Data are presented as number of *nur77* mRNA expressing cells per PVN section (unilateral).

7.2.4. Radioimmunoassays

Plasma concentrations of ACTH were measured using a commercially available immunoradiometric kit (Euro-Diagnostica) as before. The sensitivity of the assay was 1 pg/ml and the intra-assay variation was < 10%. Plasma corticosterone concentrations were determined using a commercially available radioimmunoassay kit (ICN Pharmaceuticals) at the Max Planck Institute for Psychiatry in Munich. The sensitivity of the assay was <10 ng/ml and the intra- and inter-assay variations were < 7 and < 10%, respectively.

7.2.5. Statistical Analysis

Two way analysis of variance (2-way ANOVA) followed by Student-Newman-Keuls multiple comparison test was used to analyse the *nur77* mRNA, plasma ACTH and plasma corticosterone data. P values less than 0.05 were considered statistically significant.

7.3. Results

7.3.1. Effects of forced swimming on plasma ACTH concentrations

Basal plasma concentrations of ACTH did not differ between the virgin and pregnant groups (figure 7.1.). Exposure to forced swimming evoked a significant increase in plasma ACTH concentration in both the virgin ($p < 0.001$) and the late pregnant group ($p<0.03$; two-way ANOVA), however this response was significantly attenuated in the pregnant mice (5.2-fold increase in the virgin group compared with a 2-fold increase in the pregnant group; figure 7.1.)

7.3.2. Effects of forced swimming on plasma corticosterone concentrations

Plasma corticosterone concentration was significantly higher under basal conditions in the pregnant group compared with the virgin group (figure 7.2.). Following forced swimming corticosterone levels were significantly increased in the virgin group (9.3-fold increase), however in the late pregnant group plasma corticosterone concentration was significantly lower following 10 min forced swimming compared with control levels, but not different from the post-swim concentration in virgin mice (figure 7.2.).

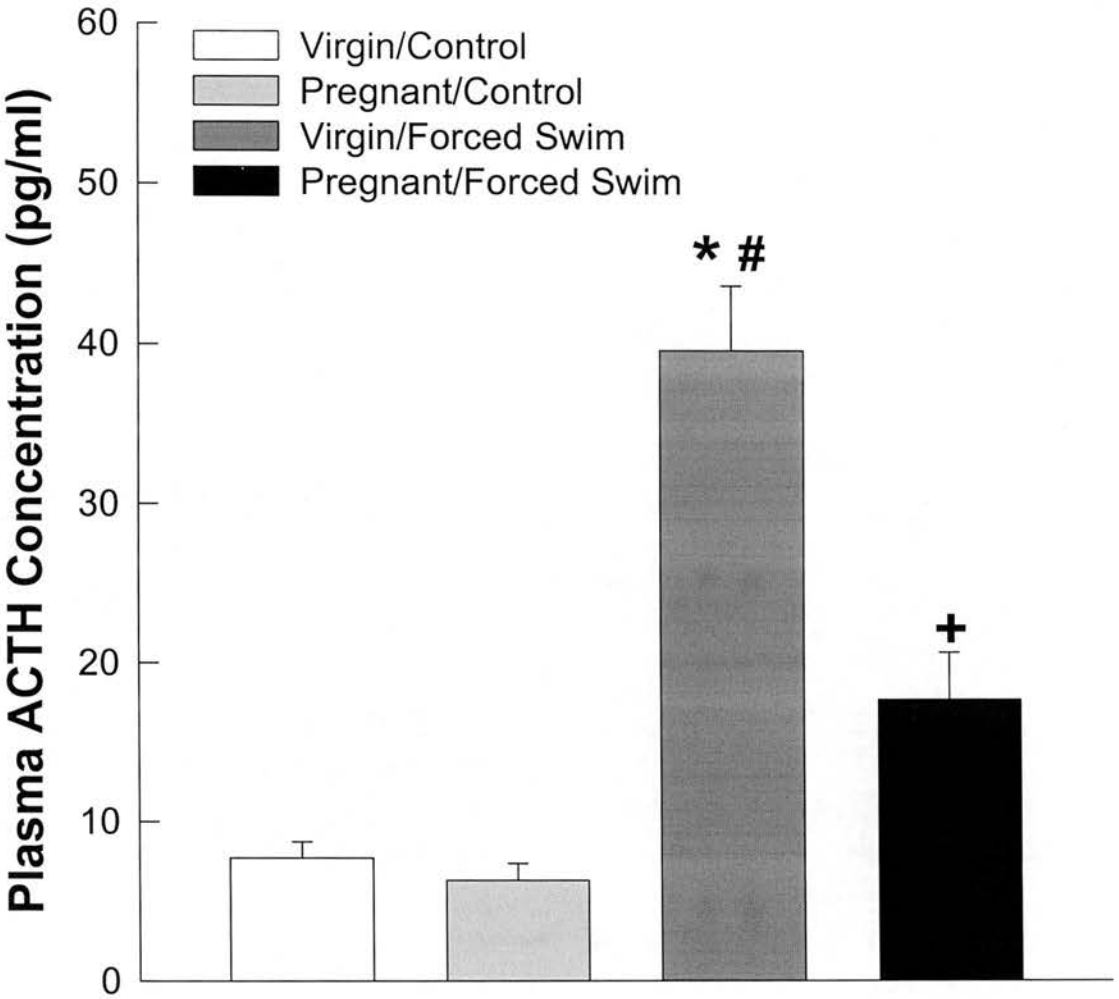


Figure 7.1. Effects of forced swimming on plasma ACTH concentrations in virgin and pregnant mice.

Virgin and pregnant mice were exposed to 10 min forced swimming in water at 17°C. Immediately after exposure to the stressor mice were decapitated and trunk blood was collected. Plasma ACTH concentration was determined using an immunoradiometric assay. Values are plotted as group means ± SEM. Group numbers: virgin/control, n = 11; pregnant/control, n = 10; virgin/forced swim, n = 11; pregnant/forced swim, n = 4. Two-way ANOVA followed by Student Newman-Keuls multiple comparison test was used for statistical analysis of the data: *$p < 0.001$ vs basal values in the same group; +$p < 0.03$ vs basal values in the same group; #$p < 0.001$ vs all other groups.

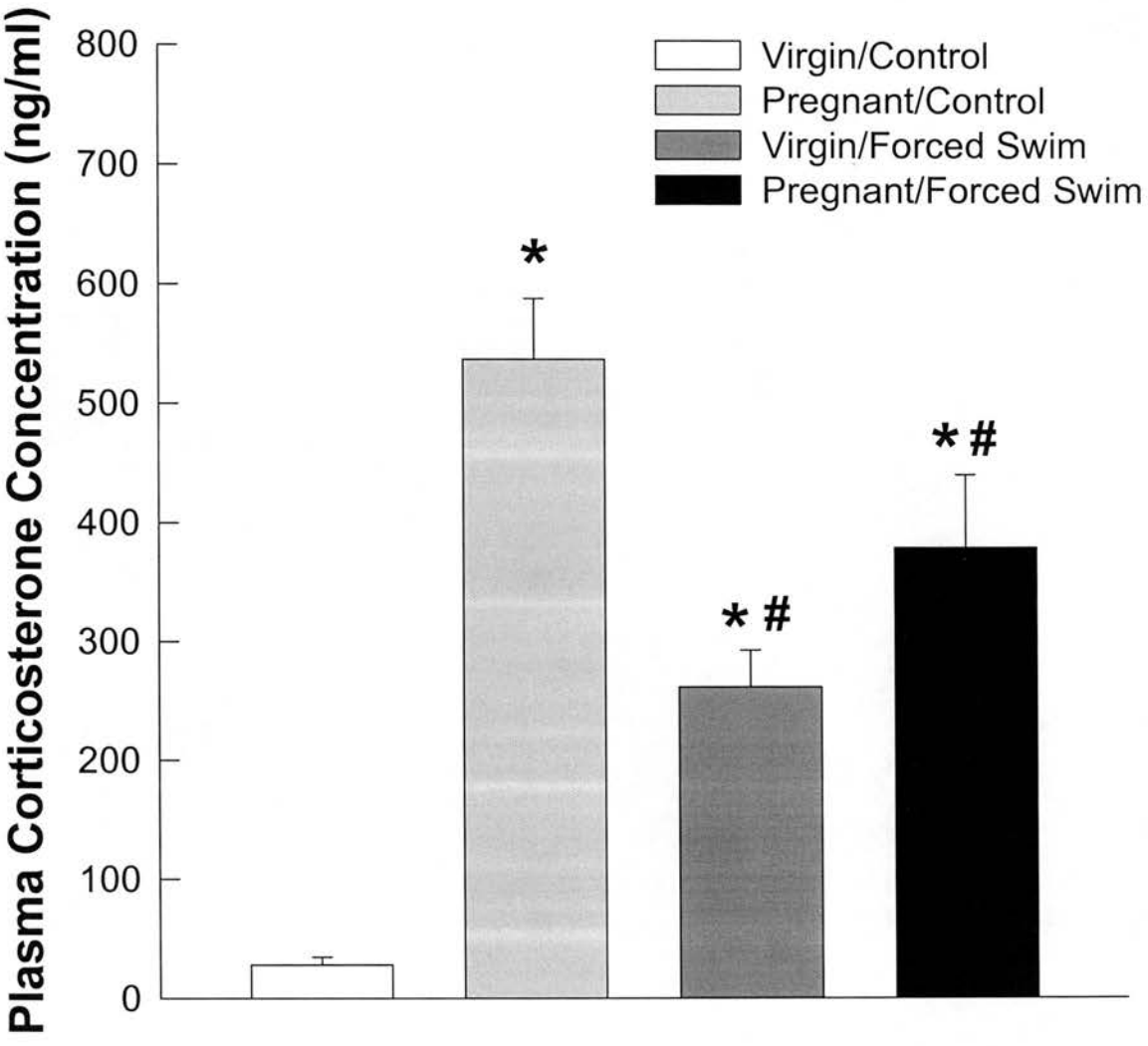


Figure 7.2. Effects of forced swimming on plasma corticosterone concentrations in virgin and pregnant mice.

Virgin and pregnant mice were exposed to 10 min forced swimming in water at 17°C. Immediately after exposure to the stressor mice were decapitated and trunk blood was collected. Plasma corticosterone concentration was determined by radioimmunoassay. Values are plotted as group means ± SEM. Group numbers: virgin/control, n = 11; pregnant/control, n = 10; virgin/forced swim, n = 11; pregnant/forced swim, n = 4. Two-way ANOVA followed by Student Newman-Keuls multiple comparison test was used for statistical analysis of the data: *$p < 0.001$ vs basal values in the virgin group; #$p < 0.02$ vs pregnant control group.

7.3.3. Effects of confinement in a glass jar on plasma ACTH concentrations

Plasma ACTH concentrations were not significantly different between the virgin and pregnant control groups (figure 7.3.). Following confinement in a jar for 10 min the virgin group demonstrated a significant increase in ACTH secretion (3.7-fold; $p<0.001$, two-way ANOVA). ACTH secretion in the pregnant group also showed a tendency to increase (2-fold), however this increase was not significant (figure 7.3.).

7.3.4. Effects of confinement in a glass jar on plasma corticosterone concentrations

As already described above plasma corticosterone concentrations were significantly higher in the pregnant group under basal conditions compared with the virgin group (figure 7.4.). Confinement in the jar for 10 min evoked a significant increase in corticosterone secretion in the virgin group, however this stressor failed to have any effect in the pregnant group (the difference between control and post-stress plasma corticosterone concentrations in the virgin group was 211.8 ± 25.3 ng/ml vs 67.6 ± 78.5 ng/ml in the pregnant group).

7.3.5. Effects of confinement in a glass jar on *nur77* mRNA expression in the pPVN

Quantification of emulsion-dipped sections revealed that *nur77* mRNA expression in the pPVN was not significantly different between the virgin and the pregnant groups under basal conditions (figure 7.5.). Confinement to the jar (10 min) evoked a significant increase in *nur77* mRNA expression in the virgin group, however this response was absent in the pregnant group (53.4 ± 3.7 vs 35.4 ± 3.5 positive cells, respectively; figure 7.5.). The level of *nur77* mRNA expression in the PVN was 1.5-fold greater in the virgin/stress group compared with the pregnant/stress group (figure 7.5.).

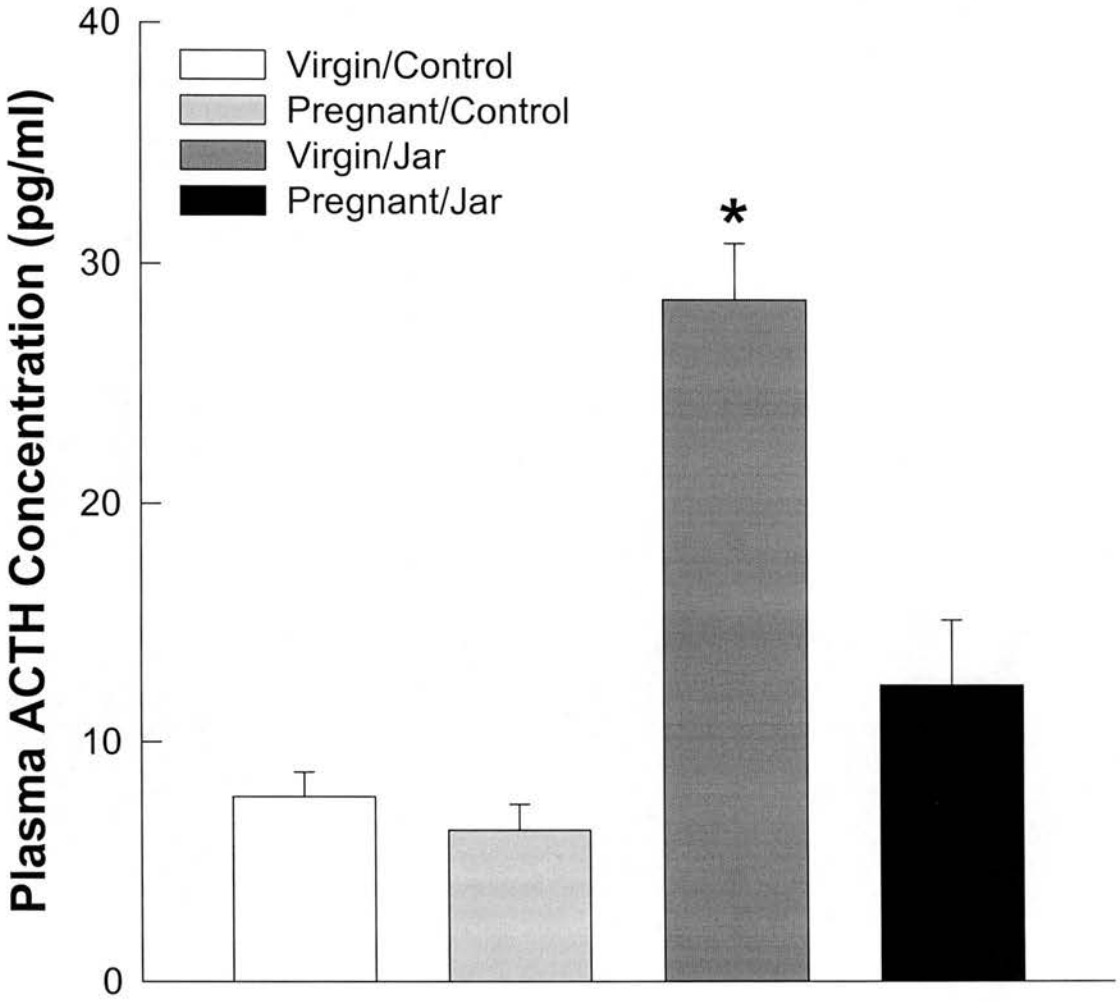


Figure 7.3. Effects of confinement in a glass jar on plasma ACTH concentrations in virgin and pregnant mice.

Virgin and pregnant mice were exposed to 10 min confinement in a glass jar. Immediately after exposure to the stressor mice were decapitated and trunk blood was collected. Plasma ACTH concentration was determined by an immunoradiometric assay. Values are plotted as group means ± SEM. Group numbers: virgin/control, n = 11; pregnant/control, n = 10; virgin/jar, n = 11; pregnant/jar, n = 9. Two-way ANOVA followed by Student Newman-Keuls multiple comparison test was used for statistical analysis of the data: *$p < 0.001$ vs all other groups.

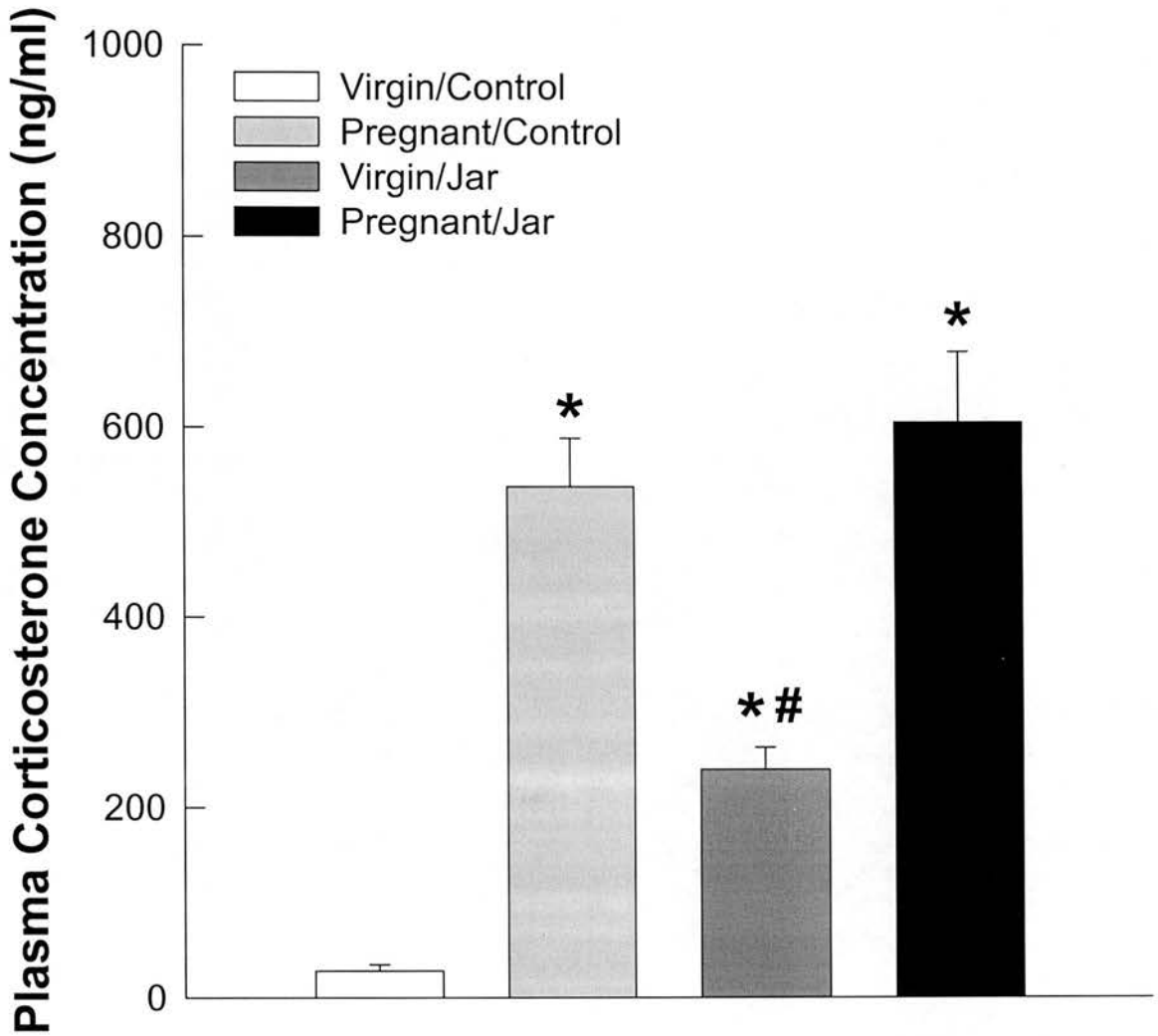


Figure 7.4. Effects of confinement in a glass jar on plasma corticosterone concentrations in virgin and pregnant mice.

Virgin and pregnant mice were exposed to 10 min confinement in a glass jar. Immediately after exposure to the stressor mice were decapitated and trunk blood was collected. Plasma corticosterone concentration was determined by radioimmunoassay. Values are plotted as group means ± SEM. Group numbers: virgin/control, n = 11; pregnant/control, n = 10; virgin/jar, n = 11; pregnant/jar, n = 9. Two-way ANOVA followed by Student Newman-Keuls multiple comparison test was used for statistical analysis of the data: *$p < 0.001$ vs basal values in the virgin group; #$p < 0.001$ vs all other groups.

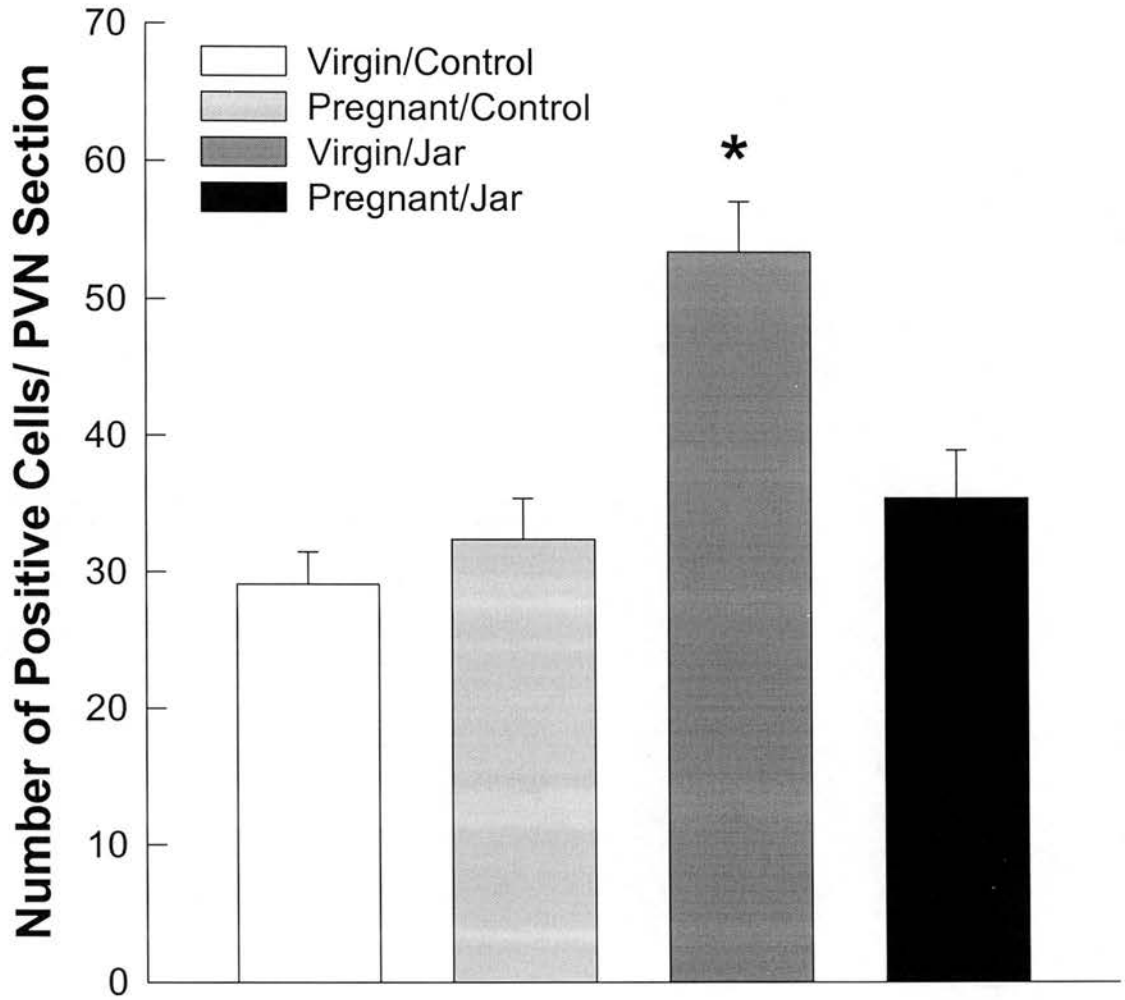
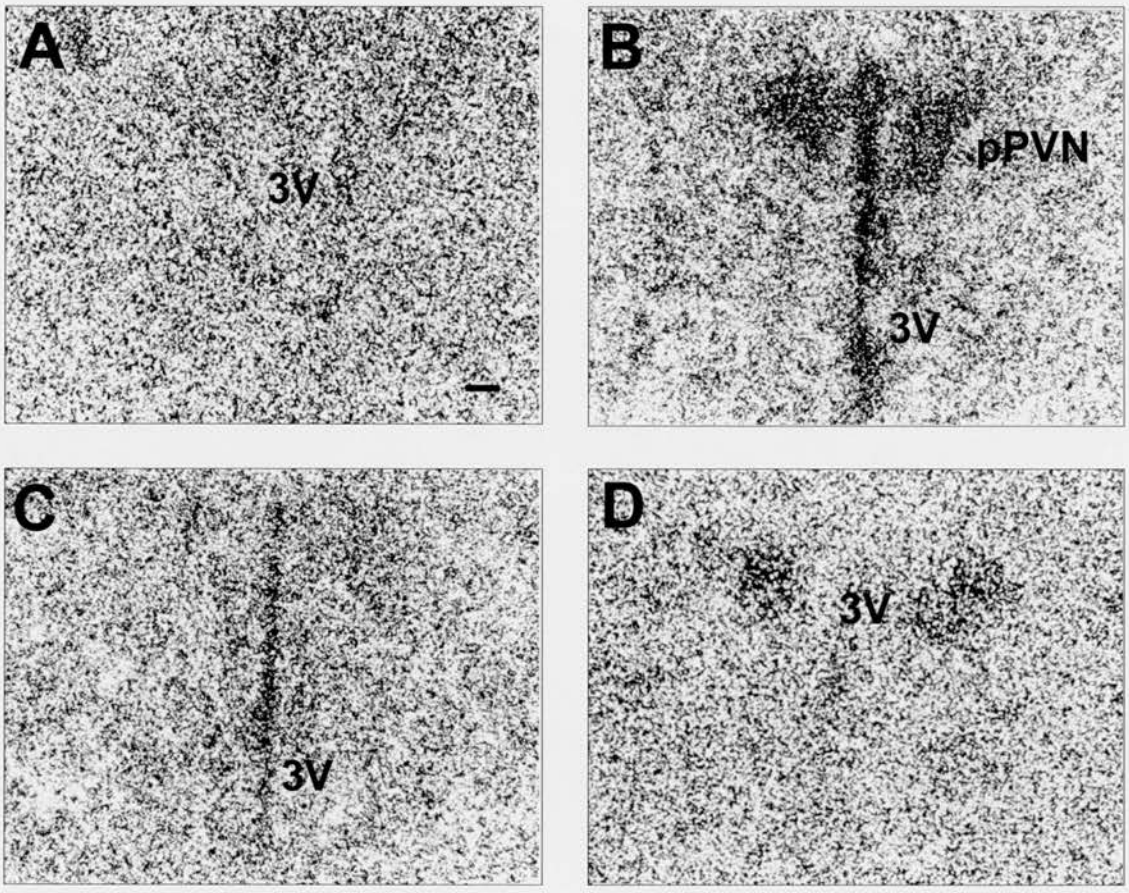


Figure 7.5.(a) Effects of confinement in a glass jar on *nur77* mRNA expression in the pPVN in virgin and pregnant mice.

Virgin and pregnant mice were exposed to 10 min confinement in a glass jar. Immediately after exposure to the stressor mice were decapitated and brains were rapidly removed and frozen on dry ice. Brain sections (15μm) were hybridised with an [^{35}S] oligo-probe complementary to *nur77* mRNA. Autoradiographs were analysed and data are presented as number of positive cells (expressing *nur77* mRNA) per PVN section (unilateral). Values are plotted as group means ± SEM. Group numbers: virgin/control, n = 9; pregnant/control, n = 9; virgin/jar, n = 13; pregnant/jar, n = 8. Two-way ANOVA followed by Student Newman-Keuls multiple comparison test was used for statistical analysis of the data: *$p < 0.001$ vs all other groups.



<u>Figure 7.5.(b)</u> Effects of confinement in a glass jar on *nur77* mRNA expression in the pPVN in virgin and pregnant mice: Photomicrographs.

Brightfield photographs of autoradiographic film exposed for 28 days to coronal brain sections hybridised with a ^{35}S-labelled oligo-probe complementary to *nur77* mRNA from: A, virgin/control; B, virgin/jar; C, pregnant/control; D, pregnant/jar. 3V, third ventricle; pPVN, parvocellular division of PVN. Scale bar = 100μm.

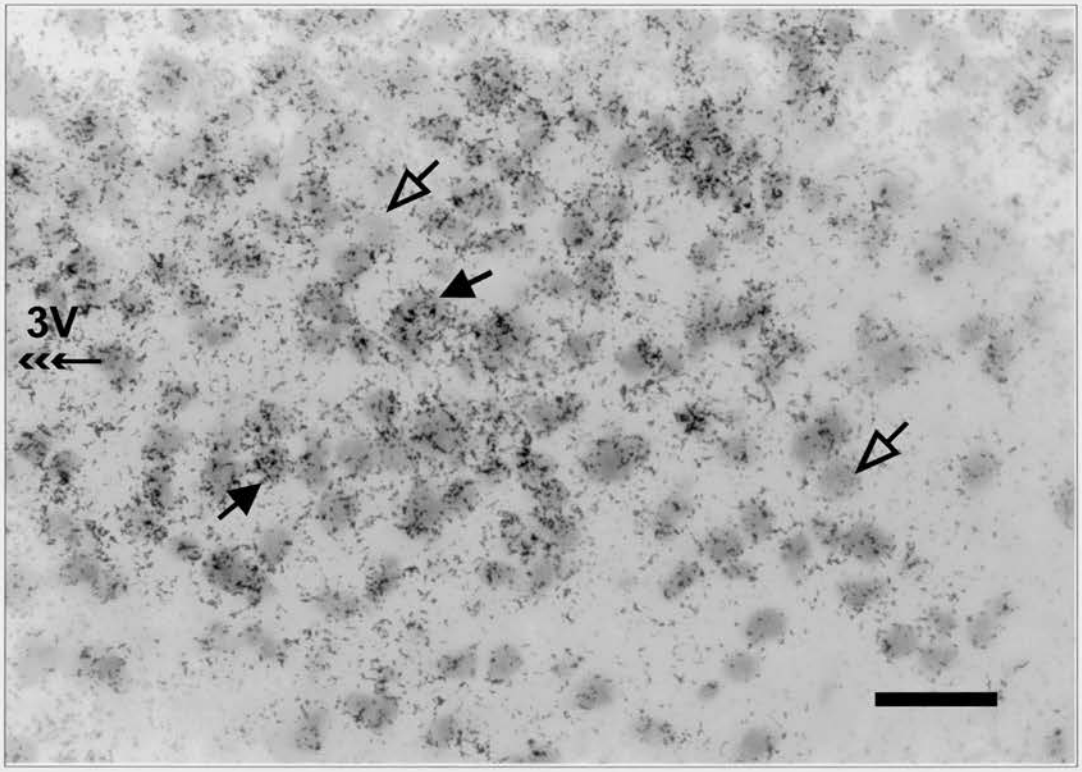


Figure 7.5.(c) Effects of confinement in a glass jar on *nur77* mRNA expression in the pPVN: Photograph of Cellular Distribution.

Brightfield photograph of an emulsion-dipped section hybridised with a ^{35}S-labelled oligo-probe complementary to *nur77* mRNA showing cellular distribution in the pPVN. This is a representative section from a virgin mouse confined to a jar for 10 minutes. The solid arrows indicate *nur77* mRNA expressing cells ("positive cells") and the open arrows indicate cells which are not expressing *nur77* mRNA. 3V indicates direction of third ventricle. Scale Bar: 50 μm.

7.4. Discussion

The present experiments demonstrate that ACTH secretion in response to confinement in a glass jar (emotional stressor) and forced swimming (a combined emotional and physical stressor) is increased in female mice as it is to other stressors in male mice (Delrue-Perollet *et al* , 1995; Chesnokova *et al* , 1998; Dunn & Swiergiel, 1999; Neveu & Liege, 2000). In pregnant mice the ACTH secretory response to both of these stressors was significantly attenuated, consistent with the findings in pregnant rats (Chapter 3-6). The attenuated ACTH secretory response in the pregnant mice seems to be a result of reduced drive by CRH and/or AVP since the parvocellular PVN neurones were activated less (indicated by reduced activation of *nur77* mRNA expression) following confinement to the jar in the pregnant group than in the virgin group.

Nur77 binds to a response element (NBRE) in the promoter region of both the CRH and the POMC gene and can regulate CRH and POMC gene transcription (Murphy *et al* , 1995; Philips *et al* , 1997). In the pregnant mice *nur77* mRNA expression was markedly reduced following exposure to stress compared with the virgin mice. Thus in pregnancy reduced activation of *nur77* in response to stress may be involved in reduced activation of the HPA axis. However studies with mice which carry a targeted mutation in the *nur77* gene (*nur77* -/-) respond normally to changes in HPA axis stimulation (with LPS, metyrapone and dexamethasone) (Crawford *et al*, 1995). One explanation is that a functionally redundant transcription factor, such as nurr1 may compensate for the loss of *nur77* in these mice. *Nurr1* encodes a product which is highly homologous to *nur77* and modulates gene transcription through the same response element, NBRE (Wilson *et al*, 1993a).

Plasma corticosterone concentrations were significantly elevated in pregnancy under basal conditions. This was not a consequence of enhanced ACTH signalling in pregnancy, since plasma ACTH concentrations were not significantly different between the virgin and pregnant groups. It may be a that sensitivity of the adrenal

gland to circulating ACTH increases during pregnancy in mice. This may be an effect of the priming effects of oestrogen on the adrenal gland, as has previously been described in rats (Carr *et al*, 1981; Burgess & Handa, 1992). Basal levels of circulating corticosterone are increased over the last few days of pregnancy in rats (Atkinson & Waddell, 1995) and cortisol levels have been shown to increase in the last trimester of pregnancy in women (Magiakou *et al*, 1996) and in late pregnant sheep (Keller-Wood & Wood, 1991). However, the rise in plasma corticosterone concentration observed here in the pregnant mice is much greater than levels normally observed in rats (1.3-fold increase in rats compared with a massive 19-fold increase in mice).

If the adrenal gland is more sensitive to circulating basal levels of ACTH in pregnant mice, then it should follow that the adrenal gland will also be more sensitive to stress-induced increases in plasma ACTH, however this does not seem to be the case. Both forced swimming and confinement to the jar induced modest increases in ACTH secretion in the pregnant mice, however this increased ACTH signalling to the adrenal gland did not result in hypersecretion of corticosterone. Instead, the increase in plasma concentrations of ACTH in response to confinement to the jar had little effect on plasma corticosterone concentrations in the pregnant mice and moreover, corticosterone secretion in response to the increase in circulating ACTH evoked by forced swimming was actually significantly reduced in the pregnant mice. This finding is difficult to explain. It may be that on exposure to stress in pregnant mice the already elevated basal levels of circulating corticosterone acts to restrain further secretion of ACTH. An alternative explanation is that the corticosterone originates from the foetal adrenals. Secretion of corticosterone by the foetal adrenals begins between day 14-15 of pregnancy in the mouse (Montano *et al*, 1993). In rats, 11β-HSD-2 would be expected to limit the passage of corticosterone across the placenta (Seckl, 1994), however in mice placental 11β-HSD-2 activity and mRNA expression are highest on day 13-14 of pregnancy but then decline progressively and are undetectable by term (Condon *et al*, 1997). Thus it is plausible that the increase in maternal plasma concentrations of corticosterone in the late pregnant mice is a consequence of increased corticosterone secretion from the foetal adrenal glands.

Whether the corticosterone detected is free or bound to CBG has not been determined as yet.

Thus in pregnant mice the responsiveness of the HPA axis to the stressors tested here is reduced at all levels, as it is during pregnancy in rats. Whether the precise mechanisms are the same in rats and mice cannot be determined from these experiments, though reduced central drive to the CRH and/or AVP neurones in response to stress is likely to play a key role in maintaining the hyporesponsiveness of the HPA axis in mice at this time. The finding that mice, as well as rats show reduced HPA axis responses to stressors (i) shows that the findings in rats are not species-specific (although the mechanisms may not be the same) and (ii) provides a foundation for exploration of the mechanisms involved using transgenic mice.

CHAPTER 8

General Discussion

General Discussion

Remarkable physiological adaptations occur in pregnancy including changes in metabolism, feeding behaviour and the cessation of ovarian cycling. The responsiveness of the HPA axis and magnocellular oxytocin neurones to stress is also altered in pregnancy. Previous studies have demonstrated that responsiveness of the HPA axis to stress is decreased progressively as term approaches (Neumann *et al*, 1998) and persists through lactation until weaning (Windle *et al*, 1997). In lactation the suckling stimulus is important for maintenance of reduced neuroendocrine responses to stress, since the responsiveness of the HPA axis returns to "normal" within two days of removal of the pups from their mother (Lightman & Young, 1989). The factor(s) involved in initiating reduced HPA axis responses in late pregnancy are not known (possibilities are discussed later) and the mechanisms underlying these attenuated responses are not fully understood. Changes at the adenohypophysis, including reduced reactivity of pituitary corticotrophs to CRH and decreased CRH receptor density (Neumann *et al* , 1998) have been shown to play a role, however other mechanisms are likely to be involved. The aim of this thesis was to investigate changes in central mechanisms underlying reduced stress responses in pregnancy, with particular focus at the level of the hypothalamus.

In Chapter 3, experiments were designed to test whether the HPA axis is less responsive to other emotional stressors in pregnancy and to establish whether these attenuated HPA axis responses to stress in pregnancy are a consequence of reduced activation of parvocellular PVN neurones. Pregnant rats demonstrated attenuated ACTH and corticosterone secretory responses to maternal aggression (see figures 3.5. and 3.6.) and reduced ACTH secretion after exposure to restraint (see figure 3.1.). Decreased NGFI-B mRNA expression in the pPVN of late pregnant rats following restraint stress indicates that the pPVN neurones are stimulated less strongly by the stressor in pregnancy (see figure 3.2.). Since a NGFI-B response element has been identified in the promoter region of the CRH gene (Wilson *et al*, 1991), NGFI-B is a putative regulator of CRH gene transcription in late pregnant

rats. Thus exposure to restraint stress is less effective in activating CRH neurones, and hence CRH gene transcription in late pregnant rats. AVP hnRNA expression in the pPVN was also significantly lower in the pregnant rats after restraint stress (see figure 3.4.). Together the data indicate that reduced activation of the HPA axis following exposure to an emotional stressor in pregnancy is a consequence of reduced stimulation of CRH and/or AVP neurones in the pPVN.

In Chapter 4 experiments were performed to investigate whether the HPA axis is also less responsive to peripheral immune challenge in pregnancy. The ACTH secretory response to i.v. LPS was strongly attenuated in pregnant rats (see figure 4.2.). Similarly there was a striking reduction in ACTH and corticosterone secretion in response to systemic IL-1β in the late pregnant rats (see figures 4.3. and 4.4.). These findings appear to be a result of reduced CRH and/or AVP drive to the anterior pituitary, since CRH mRNA expression in the pPVN was not stimulated by i.v. IL-1β in the pregnant rats, as it was in the virgin rats (see figure 4.6.), indicating failure of IL-1β to activate the CRH neurones in pregnancy. If it is assumed that activation of CRH gene transcription reflects stimulation of the CRH cell bodies, which simultaneously triggers CRH release from the nerve terminals, then it follows that less CRH is released at the median eminence in the pregnant group in response to stimulation with i.v. IL-1β. The differences in activation of the pPVN neurones between virgin and pregnant rats following systemic IL-1β are not as a result of differential activation of the brainstem input to the PVN from the A2 cell region of the NTS, since Fos expression in this region was similar in the virgin and pregnant groups after IL-1β administration (see figure 4.7.). These data indicate that signalling between the NTS and the pPVN following IL-1β administration is interrupted in pregnancy, resulting in reduced activation of the HPA axis. The precise mechanism involved is not clear though several possibilities exist (these are summarised in figure 8.1.).

Ascending noradrenergic projections from the NTS in the brainstem to the PVN are important in relaying information from the periphery to the PVN neurones following activation of the immune system (Wilson *et al* , 1991). If NA release is blocked pre-

or post-synaptically in pregnancy, this may explain the reduced responsiveness of the HPA axis to immune challenge with i.v. IL-1β. Furthermore, signalling via limbic forebrain structures including the CeA and the BNST has recently been shown to be important (Buller *et al*, 2001) in mediating activation of the HPA axis following systemic administration of IL-1β. This signalling pathway was not studied here, however interruption of signalling through these structures may contribute to the hyporesponsiveness of the HPA axis in pregnancy.

Endogenous opioids are involved in restraining HPA axis responses to IL-1β in pregnancy, since blocking the actions of opioids with the opioid receptor antagonist, naloxone, largely reinstated an ACTH secretory response to systemic IL-1β (see figure 4.8.). Moreover, naloxone pretreatment restored a response to i.v. IL-1β at the level of the CRH neurones in the pregnant rats, as indicated by increased pPVN CRH mRNA expression. However naloxone resulted in an attenuated pPVN CRH mRNA response to IL-1β in the virgin group, resulting in CRH mRNA responses that were not different between virgin and pregnant rats (see figure 4.10.). These data indicate that the effects of endogenous opioids on the HPA axis switch from being *excitatory* in virgin rats to being *inhibitory* in pregnant rats and strongly support a role for endogenous opioids in attenuated HPA axis responses to peripheral administration of IL-1β. It is feasible that endogenous opioids may be acting at the level of the noradrenergic nerve terminals to restrain NA release and thus limit excitatory input to the pPVN.

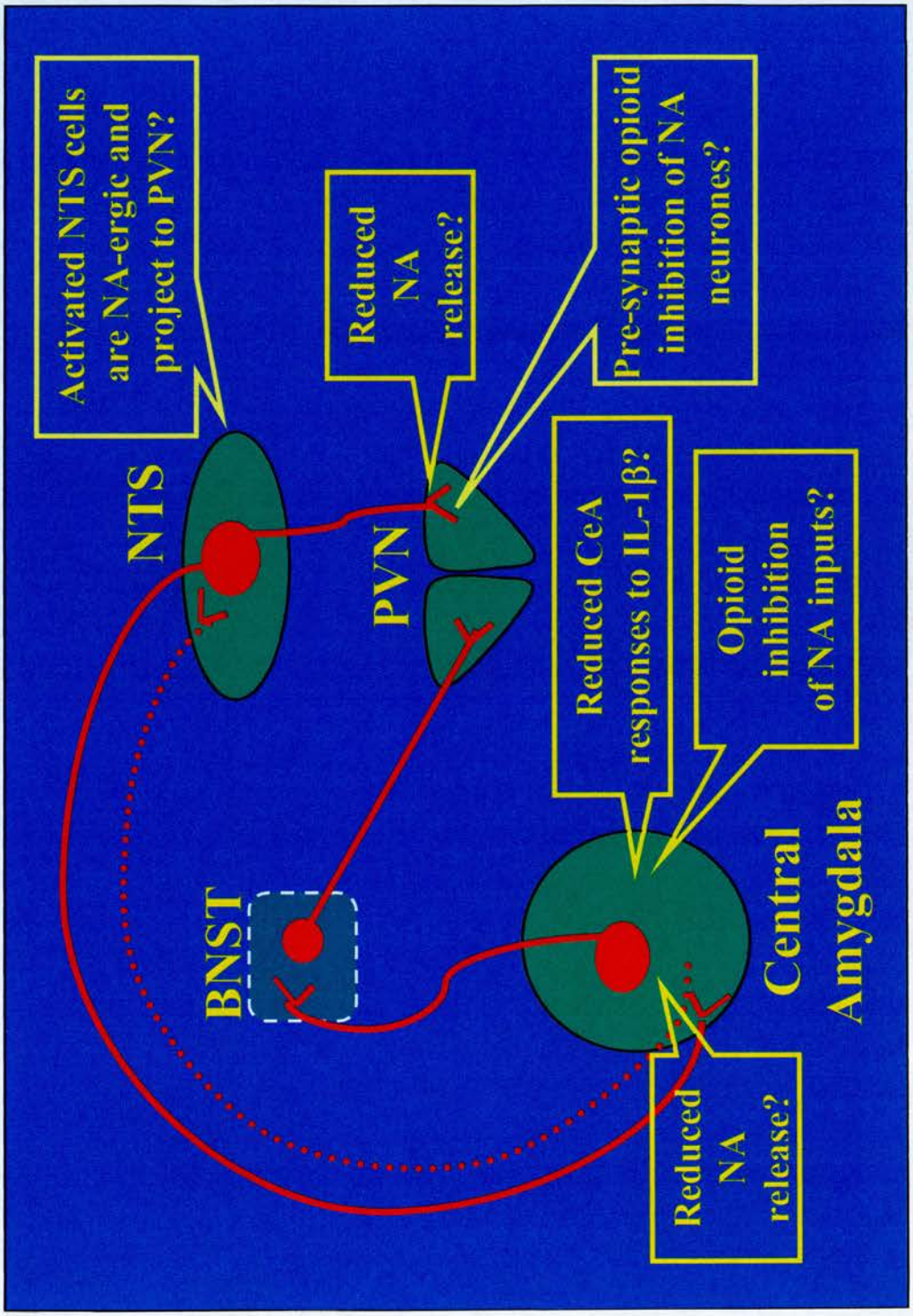


Figure 8.1. Possible mechanisms involved in reduced activation of the CRH neurones by IL-1β in pregnancy.

The oxytocin secretory response to immune challenge with systemic IL-1β was also markedly less in the pregnant group compared with the virgin group. However pretreatment with naloxone revealed an exaggerated oxytocin secretory response to IL-1β in the pregnant rats (see figure 4.9.), indicating that in pregnancy, oxytocin secretion following immune challenge is restrained by endogenous opioids. This may be an effect of opioids acting either directly on the oxytocin cell bodies or on their presynaptic inputs, possibly their noradrenergic inputs. Previous studies using cholecystokinin (CCK) to stimulate the noradrenergic projection from the A2 cell group in the brainstem NTS to oxytocin neurones have indicated that central opioid inhibition of this system is enhanced in late pregnancy (Douglas *et al*, 1995). Furthermore, naloxone potentiates the CCK-induced firing rate of oxytocin neurones in late pregnant rats but has no effect on basal activity suggesting that endogenous opioids are acting presynaptically to restrain activity of the oxytocinergic system and thus oxytocin release.

Further studies employing specific receptor antagonists are necessary to establish the specific receptor type involved in mediating the inhibition over the CRH and oxytocin neurones. Also combined immunohistochemical and retrograde tracing studies are necessary to establish the origin of the endogenous opioids responsible for restraining activity of the CRH and oxytocin neurones. Two candidate sites are the arcuate nucleus and the NTS. The arcuate nucleus is home to a population of β-endorphin cells which project to both the PVN (Drolet *et al*, 2001) and the SON (personal communication with S.L. Meddle). The number of arcuate neurones expressing POMC and β-endorphin is significantly increased by day 21 of pregnancy (Redmond *et al*, 1996). Furthermore, expression of pro-enkephalin (pENK) and μ-opioid receptor mRNA increases in the NTS neurones in late pregnancy (Meddle *et al*, 2001). Hence opioids from the arcuate nucleus and/or the NTS may be involved in modulating the activation of CRH and/or oxytocin neurones by stress in pregnancy.

Thus in pregnancy, the responsiveness of the HPA axis to the physical stressor, immune challenge is greatly reduced as it is following exposure to emotional stressors. To determine whether the CRH neurones were also non-responsive to other excitatory stimuli in pregnancy, specifically those involved in regulating metabolism and arousal, the effects of orexin-A on HPA activity was measured. I.c.v. administration of orexin-A evoked an increase in PVN CRH mRNA expression, ACTH and corticosterone secretion in virgin rats (see figures 5.1., 5.2., and 5.3.). However in pregnant rats, orexin-A failed to evoke any such effects. The failure of orexin-A to induce an increase in CRH mRNA expression indicates that the CRH neurones are not activated. This together with the previous findings suggests that in pregnancy there is a "global reduction" in the responsiveness of the CRH neurones (and hence the HPA axis) to all excitatory stimuli (though the mechanisms involved might not be the same for all stimuli).

In Chapter 6 evidence was sought for altered glucocorticoid feedback sensitivity in pregnancy. Removal of the glucocorticoid inhibitory feedback signal by phADX over 48h resulted in an increase in both CRH and AVP mRNA expression in the pPVN of both virgin and pregnant rats (see figure 6.1. and 6.2.), however phADX induced a significantly greater increase in CRH and AVP mRNA expression in the pregnant group under basal conditions. This suggests that in late pregnancy the slow feedback signal is enhanced or that the PVN neurones are more sensitive to slow glucocorticoid negative feedback. There was a concomitant rise in basal ACTH secretion in both the virgin and the pregnant rats following 48h phADX (see figure 6.3.), however in contrast to CRH and AVP mRNA expression in the PVN, the increase in ACTH secretion after phADX was significantly greater in the virgin group, suggesting altered sensitivity of the anterior pituitary to CRH and/or AVP in pregnancy. Alternatively, despite the increase in pPVN CRH and AVP mRNA expression following removal of glucocorticoid feedback it is possible that reduced forward drive still restrains CRH and/or AVP secretion in pregnancy, resulting in reduced ACTH secretion.

The ACTH and corticosterone secretory response normally observed in response to restraint stress was absent in both the virgin and the pregnant rats following 24h phADX (see figure 6.9. and 6.10). Whether this was a result of the rats being non-responsive and unaware of their environment or a consequence of a build-up of neuroactive steroids (as a result of blocking the final step in corticosterone biosynthesis, and the consequent build-up in corticosterone precursors) which may affect HPA activity is not clear, though it is an unexpected result of the phADX process.

Enhancing the rapid negative feedback signal with acute administration of exogenous corticosterone resulted in a dramatic reduction in stress-induced (forced swimming) ACTH secretion in intact virgin rats, whereas corticosterone administration had no effect in the pregnant group (see figure 6.12.). The results are consistent with a loss of rapid corticosteroid feedback in pregnancy. Thus it seems that enhanced rapid negative feedback is not involved in the reduced responsiveness of the HPA axis to acute stress in pregnancy, instead it seems more likely that if corticosterone feedback is involved it is a consequence of more prolonged exposure to elevated corticosterone levels associated with slow feedback mechanisms. Increased sensitivity to slow glucocorticoid negative feedback in pregnancy has previously been suggested from a study demonstrating increased GR mRNA expression in the dentate gyrus and increased 11β-HSD activity in the PVN on day 21 of pregnancy, concomitant with reduced levels of basal CRH and AVP mRNA expression in the PVN (Johnstone *et al*, 2000a).

Finally a comparative study was performed to determine whether the HPA axis is also hyporesponsive in another rodent species, namely the mouse. Forced swim stress and confinement to a glass jar evoked significant increases in ACTH secretion in the virgin mice, an effect that was markedly attenuated in the late pregnant mice (see figure 7.1. and 7.3.). The reduced pituitary response to these stressors in the pregnant mice is likely to be an effect of reduced drive by CRH and/or AVP (like it is in rats), since the pPVN neurones were activated by the stress less in the pregnant mice (indicated by reduced *nur 77* mRNA expression in this region; see figure 7.5.).

The greatly enhanced basal levels of plasma corticosterone compared with those in rats, suggests that other mechanisms (not present in rats, possibly involving rapid glucocorticoid feedback mechanisms) may contribute to attenuated HPA axis responses to stress in mice.

What is it about pregnancy that triggers these central changes?

Pregnancy is accompanied by a large increase in progesterone and oestrogen secretion, which in the rat are produced by the ovaries. The ovaries are essential for the maintenance of pregnancy and the collapse in progesterone secretion on the last two days of pregnancy is an important trigger for parturition (Csapo & Wiest, 1969).

Studies have shown that sex steroids can modify the stress response. Opioid inhibition of oxytocin secretory responses to stress is greater in female than male rats (Carter *et al*, 1986). The development of the endogenous opioid tone in pregnancy may partly be due to the concomitant rise in plasma oestrogen and progesterone. Progesterone increases β-endorphin content of the hypothalamus and enhances POMC expression in the arcuate nucleus (Bridges & Ronsheim, 1987), similar to that observed in pregnancy (Redmond *et al* , 1996).

Virgin rats implanted subcutaneously with oestrogen and progesterone (over 17 days to mimic levels observed in pregnancy (Bridges, 1984)) demonstrate a modest decrease in the oxytocin secretory response to forced swimming compared with control animals (Douglas *et al*, 2000), similar to the response observed in pregnancy (Douglas *et al*, 1998). However the oxytocin secretory response to forced swimming was strongly enhanced in sex steroid-treated virgin rats in the presence of naloxone, revealing strong opioid inhibition of oxytocin neuronal activity. Thus oestrogen and progesterone treatment induces opioid inhibition over oxytocin neurones, similar to that observed in pregnancy (Douglas *et al* , 1998).

In contrast, the ACTH and corticosterone secretory response to swim stress are not affected by sex steroids either alone or in the presence of naloxone (Douglas *et al* , 2000). These data suggest that increasing circulating sex steroids are important in inducing opioid restraint over oxytocin neurones in pregnancy, however do not appear to be sufficient to induce the increased opioid tone over HPA secretory responses to stress in pregnancy.

Another factor that may be involved in restraining the HPA axis in pregnancy is the progesterone metabolite, allopregnanolone. Allopregnanolone is a "neuroactive steroid" which is synthesised from cholesterol by glial cells within the brain (for reviews see (Paul & Purdy, 1992; Majewska, 1992)). Although the brain has the capacity to synthesise allopregnanolone the majority present in the brain is derived from circulating progesterone (Corpechot *et al*, 1993; Cheney *et al*, 1995). During pregnancy when circulating progesterone levels increase there is a concomitant rise in brain levels of allopregnanolone (Paul & Purdy, 1992), reaching peak concentrations on day 19 (Concas *et al*, 1998). Allopregnanolone can act upon neurones expressing $GABA_A$ receptors to enhance inhibitory GABA transmission (Majewska, 1992; Lambert *et al*, 1995; Rupprecht & Holsboer, 1999). This action of allopregnanolone has been shown to be important in regulating oxytocin neurones (Fenelon & Herbison, 1996; Fenelon & Herbison, 2000). Brussaard and colleagues have shown that in late pregnancy, allopregnanolone can directly enhance oxytocin neurone $GABA_A$ receptor signalling resulting in a powerful inhibitory influence upon the neurone (Brussaard *et al*, 1997; Brussaard *et al*, 1999; Brussaard & Herbison, 2000).

The effects of allopregnanolone on HPA activity in pregnancy have recently been investigated. Treatment of pregnant rats with the 5α-reductase (one of the enzymes involved in the conversion of progesterone to allopregnanolone) inhibitor, 4-MA fully reinstates an ACTH secretory response to forced swimming such that levels are not significantly different from virgin control rats (personal communication with S. Ma). These data strongly suggest a role for allopregnanolone in reduced HPA axis

responses to stress in pregnancy. Whether allopregnanolone also has a role in attenuated HPA axis responses to other stressors requires further investigation.

As well as the increased levels of sex steroids (and their metabolites) in pregnancy, there are also increased amounts of various peptide hormones in the maternal circulation, including relaxin and lactogenic hormones.

Relaxin is a polypeptide hormone produced by the corpora lutea. In the pregnant rat, circulating levels of relaxin become detectable around day 10 and steadily increase until a surge prepartum (Goldsmith *et al*, 1995). The actions of relaxin on the reproductive tract and its role in osmoregulation via actions on the central nervous system have been well documented (Kakouris *et al*, 1993; Goldsmith *et al* , 1995). Since levels of circulating relaxin progressively increase during the second half of gestation, at a time when attenuated HPA axis responses to stress are manifested in pregnant rats, relaxin is a possible candidate for initiating central changes. Relaxin secretion from the corpora lutea in pregnancy has previously been shown to be important for endogenous opioid inhibition of oxytocin neurones (Way *et al*, 1993). However, to date no study has investigated a role for relaxin in reduced HPA axis responses in pregnancy. Relaxin is a relatively large polypeptide (molecular weight is ~ 6000 Daltons) and would not be expected to cross the BBB. Instead relaxin is thought to exert its actions on the brain via circumventricular organs. Indeed autoradiographic studies have localised relaxin binding sites in the SFO and OVLT (Heine *et al*, 1997; McKinley *et al*, 1997). The SFO sends afferent projections to both the SON and the PVN (McKinley *et al*, 1996), hence it is not unreasonable to think that circulating relaxin may be capable of influencing the activity of CRH neurones via this pathway from the SFO. However relaxin binding sites have also been demonstrated in the PVN, SON, arcuate nucleus, amygdala and hippocampus (Heine *et al* , 1997; McKinley *et al* , 1997). Although it has yet to be shown, it is conceivable that the brain itself is capable of synthesising relaxin which may act at these binding sites to influence the responsiveness of CRH neurones to excitatory stimuli in pregnancy.

In rodents prolactin secretion during early pregnancy is induced by the mating stimulus and is characterised by twice daily surges (Gunnet & Freeman, 1983). At day 10 of pregnancy, pituitary prolactin secretion is inhibited when placental lactogen levels begin to increase (Voogt & De Greef, 1989). There are two forms of placental lactogen in rats (rPL) and both can bind to the prolactin receptor (Robertson *et al*, 1982). rPL-I is only present for a few days around mid-gestation (Robertson *et al* , 1982). The second, rPL-II begins to be detected in the blood shortly after the appearance of PL-I and progressively increases throughout the second half of pregnancy reaching peak levels by ~ day 20 (Robertson & Friesen, 1981). Prolactin is a fairly large polypeptide (197-199 amino acids) and would not be expected to cross the BBB, however there is evidence that systemic prolactin gains access to the CSF from where it can diffuse to numerous brain regions (Login. I.S & MacLeod, 1977; Nicholson *et al*, 1980; Kalin *et al*, 1981). It has been suggested that prolactin binding sites in the choroid plexus are involved in the transport of prolactin from the blood into the aCSF (Nicholson *et al* , 1980). Receptors for prolactin are present in the SON and PVN (Pi & Grattan, 1998) and RT-PCR studies (in the whole brain) have shown dramatic increases in expression of prolactin receptor mRNA during pregnancy (Sugiyama *et al*, 1994). In pregnancy and lactation prolactin mRNA expression in the hypothalamus is significantly increased (Torner *et al*, 2001). Furthermore, antagonising the prolactin receptor with i.c.v. antisense oligonucleotides increases stress-induced ACTH secretion in lactating rats (Torner *et al* , 2001). In light of the evidence above it seems possible that prolactin may play a role in the adaptations of HPA axis responses that occur in pregnancy.

What is the functional significance of these adaptations that occur in pregnancy?

Pregnancy clearly represents a conflict between the mother's need to maintain her own health and meet the demands from the developing offspring in order to maximise their potential. Therefore during pregnancy the mother must provide a safe environment with adequate nutrition and limit the exposure of the foetuses to damaging agents. So, are the changes in responsiveness of the HPA axis beneficial to both the mother and her offspring or does the mother compromise herself in order to protect the foetuses? Despite the presence of 11β-HSD in the placenta, glucocorticoids can still gain access to the foetus. In pregnancy the hyporesponsiveness of the HPA axis to stressors limits exposure of the foetus to excessive levels of corticosterone *in utero*. As discussed in Chapter 1 the developing foetus can be programmed by glucocorticoids which can have life-long detrimental consequences and predispose the offspring to various diseases throughout their life (see Chapter 1).

Restraining the maternal HPA axis may also have metabolic advantages to the pregnant mother. During late pregnancy rats increase their food intake during the dark phase (Cripps & Williams, 1975) and unsurprisingly this is associated with an increase in maternal fat and body weight (Shirley, 1984). Plasma leptin concentration increases in the dark phase, however it has recently been reported that there is no further increase in pregnant rats, as would be expected due to the increase in adipose tissue in pregnancy. Furthermore, in pregnancy there appears to be a resetting of the central mechanisms regulating appetite, since central leptin administration is less effective in reducing food intake in late pregnant rats (Johnstone & Higuchi, 2001). Thus in pregnancy reduced sensitivity to central actions of leptin will result in a decreased satiety effect and therefore produce a state of hyperphagia, thus allowing for adequate energy intake to meet the increasing demands of the foetus. Since glucocorticoids are catabolic compounds it would seem that limiting their production in pregnancy in response to stress would be beneficial to the mother. This in

association with increased food intake and reduced actions of leptin will help maintain a positive energy balance.

Here the responsiveness of the HPA axis to orexin-A was markedly attenuated in late pregnancy. No direct measure of food intake was made (as the experiment was performed early in the light phase when feeding is usually minimal), however, crude observations indicated increased "oral motor activity" in both the virgin and the pregnant orexin-treated rats. This suggests that the pregnant rats are still able to respond behaviourally to the orexigenic signal, however the pPVN neurones are stimulated less by this signalling in pregnancy. It is not known whether in pregnancy the centrally projecting CRH neurones (that project to feeding centres) respond in a similar way to those which project to the median eminence. It would be beneficial to the mother for these neurones to also be less responsive to excitatory stimuli since centrally acting CRH is known to inhibit food intake (Schwartz *et al*, 2000).

In conclusion, the responsiveness of the HPA axis to stressors is dramatically attenuated in pregnancy. The reduced responses observed in late pregnancy seem to persist irrespective of the nature of the stressor, be it a mild 'emotional' stressor or a more severe 'physical' stressor, indicating a "global reduction" in the responsiveness of the HPA axis to stress in pregnancy. Changes in central drive to the pPVN CRH/ AVP neurones and their responsiveness to excitatory inputs seems to be the most important factors in the reduced responsiveness of the HPA axis to stressors in late pregnancy, although enhanced slow glucocorticoid negative feedback may also contribute. What triggers these changes to occur is not known, however increased levels of pregnancy associated hormones and neuroactive steroids may be involved. This remarkable phenomenon, peculiar to pregnancy, will limit exposure of the foetuses to excessive levels of glucocorticoids, protect them from permanent behavioural and neuroendocrine programming and minimise their susceptibility to disease.

REFERENCES

References

Abe, K. and Critchlow, V. Delayed feedback inhibition of stress-induced activation of pituitary-adrenal function: Effects of varying dose, rate and duration of corticosterone administration and of telencephalon removal. *Neuroendocrinology* (1980) **31**: 349-354.

Abe, K., Kroning, J., Greer, M. A., and Critchlow, V. Effects of destruction of the suprachiasmatic nuclei on the circadian rhythms in plasma corticosterone, body temperature, feeding and plasma thyrotropin. *Neuroendocrinology* (1979) **29**: 119-131.

Agarwal, A. K., Monder, C., Eckstein, B., and White, P. C. Cloning and expression of rat cDNA encoding corticosteroid 11beta-dehydrogenase. *Journal of Biological Chemistry* (1989) **264**: 18939-18943.

Aguilera, G. Regulation of pituitary ACTH secretion during chronic stress. *Frontiers in Neuroendocrinology* (1994) **15**: 321-350.

Aguilera, G Corticotropin releasing hormone, receptor regulation and the stress response. *Trends In Endocrinology And Metabolism* (1998) **9**: 329-336.

Aguilera, G., Lightman, S. L., and Kiss, A. Regulation of the hypothalamic-pituitary-adrenal axis during water deprivation. *Endocrinology* (1993) **132**: 241-248.

Aguilera, G., Millan, M. A., Hauger, R. L., and Catt, K. J. Corticotropin-releasing factor receptors: Distribution and regulation in brain, pituitary, and peripheral tissues. *Annals of the New York Academy of Sciences.* (1987) **512** : 48-66.

Aguilera, G., Rabadan-Diehl, C., and Nikodemova, M. Regulation of pituitary corticotropin releasing hormone receptors. *Peptides* (2001) **22**: 769-774.

Akana, S. F. and Dallman, M. F. Chronic cold in adrenalectomized, corticosterone (B)-treated rats: Facilitated corticotropin responses to acute restraint emerge as B increases. *Endocrinology* (1997) **138**: 3249-3258.

Akil, H., Watson, S. J., Young, E. and others. Endogenous opioids: Biology and function. *Annual Review of Neuroscience* (1984) **7**: 223-255.

Al-Barazanji, K. A., Wilson, S., Baker, J., Jessop, D. S., and Harbuz, M. S. Central orexin-A activates hypothalamic-pituitary-adrenal axis and stimulates hypothalamic corticotropin releasing factor and arginine vasopressin neurones in conscious rats *Journal of Neuroendocrinology* (2001) **13**: 421-424.

Albiston, A. L., Obeyesekere, V. R., Smith, R. E., and Krozowski, Z. S. Cloning and tissue distribution of the human 11beta-hydroxysteroid dehydrogenase type 2 enzyme. *Molecular & Cellular Endocrinology* (1994) **105**: R11-R17.

Andersson, J., Nagy, S., Bjork, L., Abrams, J., Holm, S., and Andersson, U. Bacterial toxin-induced cytokine production studied at the single-cell level. *Immunological Reviews* (1992) **127:** 69-96.

Andreis, P. G., Neri, G., Belloni, A. S., Mazzocchi, G., Kasprzak, A., and Nussdorfer, G. G. Interleukin-1beta enhances corticosterone secretion by acting directly on the rat adrenal gland. *Endocrinology* (1991) **129**: 53-57.

Antoni, F. A. Hypothalamic control of ACTH secretion: Advances since the discovery of 41-residue CRF. *Endocrine Reviews* (1986) **7**: 351-378.

Antoni, F. A. Vasopressinergic control of pituitary adrenocorticotropin secretion comes of age. *Frontiers in Neuroendocrinology* (1993) **14**: 76-122.

Antoni, F. A., Holmes, M. C., and Jones, M. T. Oxytocin as well as vasopressin potentiate ovine CRF in vitro. *Peptides* (1884) **4**: 411-415.

Antoni, F. A., Holmes, M. C., and Kiss, J. Z. Pituitary binding of vasopressin is altered by experimental manipulations of the hypothalamo-pituitary-adrenocortical axis in normal as well as homozygous (di/di) Brattleboro rats. *Endocrinology* (1985) **117**: 1293-1299.

Arase, K., York, D. A., Shimizu, H., Shargill, N., and Bray, G. A. Effects of corticotropin-releasing factor on food intake and brown adipose tissue thermogenesis in rats. *American Journal of Physiology - Endocrinology & Metabolism* (1988) **255**: 18-3.

Arborelius, L., Owens, M. J., Plotsky, P. M., and Nemeroff, C. B. The role of corticotropin-releasing factor in depression and anxiety disorders. *Journal of Endocrinology* (1999) **160**: 1-12.

Arimura, A., Saito, T., Bowers, C. Y., and Schally, A. V. Pituitary adrenal activation in rats with hereditory hypothalamic diabetes insipidus. *Acta Endocrinology* (1967) **54**: 155-65.

Armstrong, R. C. and Montminy, M. R. Transsynaptic control of gene expression. *Annual Review of Neuroscience* (1993) **16**: 17-29.

Arriza, J. L., Simerly, R. B., Swanson, L. W., and Evans, R. M. The neuronal mineralocorticoid receptor as a mediator of glucocorticoid response. *Neuron* (1988) **1**: 887-900.

Arriza, J. L., Weinberger, C., Cerelli, G. and others. Cloning of human mineralocorticoid receptor complementary DNA: Structural and functional kinship with the glucocorticoid receptor *Science* (1987) **237**: 268-275.

Atkinson, H. C. and Waddell, B. J. The hypothalamic-pituitary-adrenal axis in rat pregnancy and lactation: circadian variation and interrelationship of plasma adrenocorticotropin and corticosterone. *Endocrinology* (1995) **136**: 512-20.

Aubry, J. M., Turnbull, A. V., Pozzoli, G., Rivier, C., and Vale, W. Endotoxin decreases corticotropin-releasing factor receptor 1 messenger ribonucleic acid levels in the rat pituitary. *Endocrinology* (1997) **138**: 1621-1626.

Autelitano, D. J. Glucocorticoid Regulation Of c-fos, c-jun And Transcription Factor AP-1 In The AtT-20 Corticotrope Cell. *Journal of Neuroendocrinology* (1994) **6**: 627-637.

Autelitano, D. J Stress-induced stimulation of pituitary POMC gene expression is associated with activation of transcription factor AP-1 in hypothalamus and pituitary. *Brain Research Bulletin* (1998) **45**: 75-82.

Bagdade, J. D., Bierman, E. L., and Porte, D. Jr. The significance of basal insulin levels in the evaluation of the insulin response to glucose in diabetic and non-diabetic subjects. *Journal of Clinical Investigation* (1967) **46**: 1549-1557.

Bagdy, G., Chrousos, G. P., and Calogero, A. E. Circadian patterns of plasma immunoreactive corticotropin, beta-endorphin, corticosterone and prolactin after immunoneutralization of corticotropin-releasing hormone. *Neuroendocrinology* (1991) **53**: 573-578.

Bamberger, C. M., Schulte, H. M., and Chrousos, G. P. Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. *Endocrine Reviews* (1996) **17**: 245-261.

Baigen, S. M. and Lowry, P. J. mRNA expression profiles for corticotrophin-releasing factor (CRF), urocortin, CRF receptors and CRF-binding protein in peripheral rat tissues. *Journal of Molecular Endocrinology* (2000) **25**: 43-52.

Baldwin, H. A., Rassnick, S., Rivier, J., Koob, G. F., and Britton, K. T. CRF antagonist reverses the 'anxiogenic' response to ethanol withdrawal in the rat. *Psychopharmacology* (1991) **103**: 227-232.

Balkwill, F. R. and Burke, F. The cytokine network. *Immunology Today* (1989) **10**: 299-304.

Ban, E., Haour, F., and Lenstra, R. Brain interleukin 1 gene expression induced by peripheral lipopolysaccharide administration. *Cytokine* (1992) **4**: 48-54.

Barbazanges, A., Piazza, P. V., Le Moal, M., and Maccari, S. Maternal glucocorticoid secretion mediates long-term effects of prenatal stress. *Journal of Neuroscience* (1996) **16**: 3943-3949.

Bartanusz, V., Jezova, D., Bertini, L. T., Tilders, F. J. H., Aubry, J. M., and Kiss, J. Z. Stress-induced increase in vasopressin and corticotropin-releasing factor expression in hypophysiotrophic paraventricular neurons. *Endocrinology* (1993) **132**: 895-902.

Baskin, D. G., Breininger, J. F., and Schwartz, M. W. Leptin receptor mRNA identifies a subpopulation of neuropeptide y neurons activated by fasting in rat hypothalamus. *Diabetes* (1999) **48**: 828-833.

Baskin, D. G., Wilcox, B. J., Figlewicz, D. P., and Dorsa, D. M. Insulin and insulin-like growth factors in the CNS. *Trends in Neurosciences* (1988) **11** : 107-111.

Baura, G. D., Foster, D. M., Porte Jr, D., Kahn, S. E., Bergman, R. N., Cobelli, C., and Schwartz, M. W. Saturable transport of insulin from plasma into the central nervous system of dogs in vivo. A mechanism for regulated insulin delivery to the brain *Journal of Clinical Investigation* (1993) **92**: 1824-1830.

Beaulieu, S., Di Paolo, T., and Barden, N. Control of ACTH secretion by the central nucleus of the amygdala: Implication of the serotoninergic system and its relevance to the glucocorticoid delayed negative feedback mechanism. *Neuroendocrinology* (1986) **44**: 247-254.

Beaulieu, S., Di Paolo, T., Cote, J., and Barden, N. Participation of the central amygdaloid nucleus in the response of adrenocorticotropin secretion to immobilization stress: Opposing roles of the noradrenergic and dopaminergic systems. *Neuroendocrinology* (1987) **45**: 37-46.

Beesley, J. E. Immunocytochemistry and In Situ Hybridisation in the Biomedical Sciences. *Published by Birkhauser* (2001)

Behan, D. P., Potter, E., Lewis, K. A., Jenkins, N. A., Copeland, N., Lowry, P. J., and Vale, W. W. Cloning and structure of the human corticotrophin releasing factor-binding protein gene (CRHBP). *Genomics* (1993) **16**: 63-68.

Bell, M. E., Bhatnagar, S., Liang, J., Soriano, L., Nagy, T. R., and Dallman, M. F. Voluntary sucrose ingestion, like corticosterone replacement, prevents the metabolic deficits of adrenalectomy. *Journal of Neuroendocrinology* (2000) **12**: 461-470.

Benediktsson, R., Burt, D., Lindsay, R. S., Seckl, J. R., and Edwards, C. R. W. Blood pressure and birth weight: Is fetal glucocorticoid exposure the missing link? *Journal of Hypertension* (1992) **10**: 1434.

Benediktsson, R., Lindsay, R. S., Noble, J., Seckl, J. R., and Edwards, C. R. W. Glucocorticoid exposure in utero: New model for adult hypertension *Lancet* (1993) **341**: 339-341.

Benediktsson, R., Noble, J., Calder, A. A., Edwards, C. R. W., and Seckl, J. R. 11-beta-HSD activity in intact dually-perfused fresh hunan placenta predicts birth weight. *Journal of Endocrinology* (1995) **144** (Suppl): P161.

Berkenbosch, F., van Oers, J., del Rey, A., Tilders, F., and Besedovsky, H. Corticotropin-releasing factor-producing neurons in the rat activated by interleukin-1 *Science* (1987) **238**: 524-6.

Bernardini, R., Kamilaris, T. C., Calogero, A. E., Johnson, E. O., Gomez, M. T., Gold, P. W., and Chrousos, G. P. Interactions between tumor necrosis factor-alpha, hypothalamic corticotropin-releasing hormone, and adrenocorticotropin secretion in the rat *Endocrinology* (1990) **126**: 2876-2881.

Bernardis, L. L. and Bellinger, L. L. The lateral hypothalamic area revisited: Neuroanatomy, body weight regulation, neuroendocrinology and metabolism. *Neuroscience & Biobehavioral Reviews* (1993) **17**: 141-193.

Bernardis, L. L. and Bellinger, L. L. The lateral hypothalamic area revisited: Ingestive behavior. *Neuroscience & Biobehavioral Reviews* (1996) **20**: 189-287.

Besedovsky, H. O. and Del Rey, A. Immune-neuroendocrine circuits: Integrative role of cytokines. *Frontiers in Neuroendocrinology* (1992) **13**: 61-94.

Bhatnagar, S., Bell, M. E., Liang, J., Soriano, L., Nagy, T. R., and Dallman, M. F. Corticosterone facilitates saccharin intake in adrenalectomized rats: Does corticosterone increase stimulus salience? *Journal of Neuroendocrinology* (2000) **12**: 453-460.

Birnberg, N. C., Lissitzky, J. C., Hinman, M., and Herbert, E. Glucocorticoids regulate proopiomelanocortin gene expression in vivo at the levels of transcription and secretion. *Proceedings of the National Academy of Sciences of the United States of America* (1983) **80**: 6982-6986.

Blalock, J. E. A molecular basis for bidirectional communication between the immune and neuroendocrine systems *Physiological Reviews* (1989) **69**: 1-32.

Bloom, F. E., Battenberg, E. L. F., Rivier, J., and Vale, W. Corticotropin releasing factor (CRF): Immunoreactive neurones and fibers in rat hypothalamus. *Regulatory Peptides* (1982) **4**: 43-48.

Borski, R. J. Nongenomic membrane actions of glucocorticoids in vertebrates. *Trends in Endocrinology & Metabolism* (2000) **11**: 427-436.

Boutillier, A. L., Monnier, D., Lorang, D., Lundblad, J. R., Roberts, J. L., and Loeffler, J. P. Corticotropin-releasing hormone stimulates proopiomelanocortin transcription by cFos-dependent and -independent pathways: Characterization of an AP1 site in exon 1. *Molecular Endocrinology* (1995) **9**: 745-755.

Bray, G. A. Hypothalamic and genetic obesity: An appraisal of the autonomic hypothesis and the endocrine hypothesis. *International Journal of Obesity* (1984) **8**: 119-137.

Bray, G. A., Fisler, J., and York, D. A. Neuroendocrine control of the development of obesity: Understanding gained from studies of experimental animal models. *Frontiers in Neuroendocrinology* (1990) **11**: 128-181.

Bridges, R. S. A quantitative analysis of the roles of dosage, sequence, and duration of estradiol and progesterone exposure in the regulation of maternal behavior in the rat. *Endocrinology* (1984) **114**: 930-940.

Bridges, R. S. and Ronsheim, P. M. Immunoreactive beta-endorphin concentrations in brain and plasma during pregnancy in rats: Possible modulation by progesterone and estradiol. *Neuroendocrinology* (1987) **45**: 381-388.

Brindley, D. N. and Rolland, Y. Possible connections between stress, diabetes, obesity, hypertension and altered lipoprotein metabolism that may result in atherosclerosis. *Clinical Science* (1989) **77**: 453-461.

Britton, D. R., Koob, G. F., Rivier, J., and Vale, W. Intraventricular corticotropin-releasing factor enhances behavioral effects of novelty. *Life Sciences* (1982) **31**: 363-367.

Broberger, C., Johansen, J., Johansson, C., Schalling, M., and Hokfelt, T. The neuropeptide Y/agouti gene-related protein (AGRP) brain circuitry in normal, anorectic, and monosodium glutamate-treated mice. *Proceedings of the National Academy of Sciences of the United States of America* (1998) **95**: 15043-15048.

Broberger, C., Visser, T. J., Kuhar, M. J., and Hokfelt, T. Neuropeptide Y innervation and neuropeptide-Y-Y1-receptor-expressing neurons in the paraventricular hypothalamic nucleus of the mouse. *Neuroendocrinology* (1999) **70**: 295-305.

Bronstein, D. M., Schafer MK-H, Watson, S. J., and Akil, H. Evidence that beta-endorphin is synthesized in cells in the nucleus tractus solitarius: Detection of POMC mRNA. *Brain Research* (1992) **587**: 269-275.

Brown, C. H., Russell, J. A., and Leng, G. Opioid modulation of magnocellular neurosecretory cell activity. *Neuroscience Research* (2000) **36**: 97-120.

Brown, R. E. Cytokines and the interaction between the neuroendocrine and immune systems. *In: An introduction to Neuroendocrinology* (1998) 302-335.

Brown, R. E. (Ed) The pituitary gland and its hormones. *An Introduction to Neuroendocrinology* (1998) 30-39.

Brown, R. W., Chapman, K. E., Edwards, C. R. W., and Seckl, J. R. Human placental 11beta-hydroxysteroid dehydrogenase: Evidence for and partial purification of a distinct NAD-dependent isoform. *Endocrinology.* (1993) **132**: 2614-2621.

Brussaard, A. B., Devay, P., Leyting-Vermeulen, J. L., and Kits, K. S. Changes in properties and neurosteroid regulation of GABAergic synapses in the supraoptic nucleus during the mammalian female reproductive cycle. *Journal of Physiology* (1999) **516**: 513-524.

Brussaard, A. B. and Herbison, A. E. Long-term plasticity of postsynaptic GABA(A)-receptor function in the adult brain: Insights from the oxytocin neurone. *Trends in Neurosciences* (2000) **23**: 190-195.

Brussaard, A. B., Kits, K. S., Baker, R. E., Willems, W. P. A., Leyting-Vermeulen, J. W., Voorn, P., Smit, A. B., Bicknell, R. J., and Herbison, A. E. Plasticity in fast synaptic inhibition of adult oxytocin neurons caused by switch in GABA(A) receptor subunit expression. *Neuron* (1997) **19**: 1103-1114.

Buckingham, J. C. The influence of corticosteroids on the secretion of corticotrophin and its hypothalamic releasing hormone. *Journal of Physiology* (1979) **286**: 331-342.

Buckingham, J. C. Secretion of corticotrophin and its hypothalamic releasing factor in response to morphine and opioid peptides. *Neuroendocrinology* (1982) **35**: 111-116.

Buckingham, J. C. Stimulation and inhibition of corticotrophin releasing factor secretion by beta endorphin. *Neuroendocrinology* (1986) **42**: 148-152.

Buckingham, J. C. and Cooper, T. A. Differences in hypothalamo-pituitary-adrenocortical activity in the rat after acute and prolonged treatment with morphine. *Neuroendocrinology* (1984) **38**: 411-417.

Buckingham, J. C. and Cooper, T. A. Effects of naloxone on hypothalamo-pituitary-adrenocortical activity in the rat. *Neuroendocrinology* (1986) **42**: 421-426.

Buckingham, J. C. and Hodges, J. R. Interrelationships of pituitary and plasma corticotrophin and plasma corticosterone in adrenalectomized and stressed, adrenalectomized rats. *Journal of Endocrinology* (1974) **63**: 213-222.

Buckingham, J. C. and Hodges, J. R. The use of corticotrophin production by adenohypophysial tissue in vitro for the detection and estimation of potential corticotrophin releasing factors. *Journal of Endocrinology* (1977) **72**: 187-193.

Buckingham, J. C., Loxley, H. D., Christian, H. C., and Philip, J. G. Activation of the HPA axis by immune insults: Roles and interactions of cytokines, eicosanoids, and glucocorticoids. *Pharmacology, Biochemistry & Behavior* (1996) **54**: 285-298.

Buhler, H., Perschel, F. H., Fitzner, R., and Hierholzer, K. Endogenous inhibitors of 11beta-OHSD: Existence and possible significance. *Steroids* (1994) **59**: 131-135.

Bull, P. M., Douglas, A. J., and Russell, J. A. Opioids and coupling of the anterior peri-third ventricular input to oxytocin neurones in anaesthetized pregnant rats. *Journal of Neuroendocrinology* (1994) **6**: 267-274.

Bull, P. M. and Russell, J. A. Oxytocin secretory responses to hypernatraemia and inhibition by morphine in pregnant urethane-anaesthetised rats. *Journal of Physiology* (1992) **452P**: 210P.

Buller, K. M., Crane, J. W., and Day, T. A. The central nucleus of the amygdala: A conduit for modulation of HPA axis responses to an immune challenge? *Stress* (2001) **4**: 277-287.

Buller, K. M., Xu, Y., Dayas, C. V., and Day, T. A. Dorsal and ventral medullary catecholamine cell groups contribute differentially to systemic interleukin-1beta-induced HPA axis responses. *Neuroendocrinology* (2001) **73**: 129-138

Buller, K. M., Xu, Y., and Day, T. A. Indomethacin attenuates oxytocin and hypothalamic-pituitary-adrenal axis responses to systemic interleukin-1beta. *Journal of Neuroendocrinology* (1998) **10**: 519-528.

Burgess, L. H. and Handa, R. J. Chronic estrogen-induced alterations in adrenocorticotropin and corticosterone secretion, and glucocorticoid receptor-mediated functions in female rats. *Endocrinology* (1992) **131**: 1261-1269.

Burlet, A., Tonon, M. C., Tankosic, P. and others Comparative immunocytochemical localization of corticotropin releasing factor (CRF-41) and neurohypophysial peptides in the brain of Brattleboro and Long-Evans rats. *Neuroendocrinology* (1983) **37**: 64-72.

Burton, P. J. and Waddell, B. J. 11beta-Hydroxysteroid dehydrogenase in the rat placenta: Developmental changes and the effects of altered glucocorticoid exposure. *Journal of Endocrinology* (1994) **143**: 505-513.

Campeau, S., Day, H. E. W., Helmreich, D. L., Kollack-Walker, S., and Watson, S. J. Principles of psychoneuroendocrinology. *Psychiatric Clinics of North America.* (1998) **21**: 259-276.

Cannon, W. B. The Autonomic Nervous System: An Interpretation. *Lancet* (1930) **1**: 1109.

Cao, C., Matsumura, K., Yamagata, K., and Watanabe, Y. Endothelial cells of the rat brain vasculature express cyclooxygenase-2 mRNA in response to systemic interleukin-1beta: A possible site of prostaglandin synthesis responsible for fever. *Brain Research* (1996) **733**: 263-272.

Cao, C., Matsumura, K., Yamagata, K., and Watanabe, Y. Cyclooxygenase-2 is induced in brain blood vessels during fever evoked by peripheral or central administration of tumor necrosis factor. *Molecular Brain Research* (1998) **56**: 45-56.

Carr, R. R., Parker, C. R., Madden, J. D., MacDonald, P. C., and Porter, J. C. Maternal plasma ACTH and cortisol relationships throughout human pregnancy. *American Journal of Obstetrics and Gynecology* (1981) **139**: 416-421.

Carter, D. A., Williams, T. D. M, and Lightman, S. L. A Sex Difference In Endogenous Opioid Regulation Of The Posterior Pituitary-Response To Stress In The Rat. *Journal of Endocrinology* (1986) **111**: 239-244.

Cascio, C. S., Shinsako, J., and Dallman, M. F. The suprachiasmatic nuclei stimulate evening ACTH secretion in the rat. *Brain Research* (1987) **423**: 173-178.

Catt, K. J., Harwood, J. P., Aguilera, G., and Dufau, M. L. Hormonal regulation of peptide receptors and target cell responses. *Nature* (1979) **280**: 109-116.

Ceccatelli, S. and Orazzo, C. Effect of different types of stressors on peptide messenger ribonucleic acids in the hypothalamic paraventricular nucleus. *Acta Endocrinologica* (1993) **128**: 485-492.

Ceccatelli, S., Seroogy, K. B., Millhorn, D. E., and Terenius, L. Presence of a dynorphin-like peptide in a restricted subpopulation of catecholaminergic neurons in rat nucleus tractus solitarii. *Brain Research* (1992) **589**: 225-230.

Champagne, F. and Meaney, M. J. Like mother, like daughter: Evidence for non-genomic transmission of parental behaviour and stress responsivity. *Progress in Brain Research* (2001) **133**:

Chan, R. K. W., Brown, E. R., Ericsson, A, Kovacs, K. J., and Sawchenko, P. E. A comparison of two immediate-early genes, c-fos and NGFI-B, as markers for functional activation in stress-related neuroendocrine circuitry. *The Journal of Neuroscience* (1993a) **13**: 5126-5138.

Chemelli, R. M., Willie, J. T., Sinton, C. M., Elmquist, J. K., Scammell, T., Lee, C., Richardson, J. A., Williams, S. C., Xiong, Y., Kisanuki, Y., Fitch, T. E., Nakazato, M., Hammer, R. E., Saper, C. B., and Yanagisawa, M. Narcolepsy in orexin knockout mice: Molecular genetics of sleep regulation. *Cell* (1999) **98**: 437-451.

Chen, C. L. C., Chang, C. C., Krieger, D. T., and Bardin, C. W. Expression and regulation of proopiomelanocortin-like gene in the ovary and placenta: Comparison with the testis. *Endocrinology* (1986) **118**: 2382-2389.

Chen, R., Lewis, K. A., Perrin, M. H., and Vale, W. W. Expression cloning of a human corticotropin-releasing-factor receptor. *Proceedings of the National Academy of Sciences of the United States of America* (1993) **90**: 8967-8971.

Cheney, D. L., Uzunov, D., Costa, E., and Guidotti, A. Gas chromatographic-mass fragmentographic quantitation of 3alpha-hydroxy- 5alpha-pregnan-20-one (allopregnanolone) and its precursors in blood and brain of adrenalectomized and castrated rats. *Journal of Neuroscience* (1995) **15**: 4641-4650.

Chesnokova, V., Auernhammer, C. J., and Melmed, S. Murine leukemia inhibitory factor gene disruption attenuates the hypothalamo-pituitary-adrenal axis stress response. *Endocrinology* (1998) **139**: 2209-2216.

Cheung, C. C., Clifton, D. K., and Steiner, R. A. Proopiomelanocortin neurons are direct targets for leptin in the hypothalamus. *Endocrinology* (1997) **138**: 4489-4492.

Childs, G. V., Morell, J. L., Niendorf, A., and Aguilera, G. Cytochemical studies of corticotropin-releasing factor (CRF) receptors in anterior lobe corticotropes: Binding, glucocorticoid regulation and endocytosis of [biotinyl-Ser1]CRF. *Endocrinology* (1986) **119**: 2129-2142.

Childs, G. V. and Unabia, G. Rapid corticosterone inhibition of corticotropin-releasing hormone binding and adrenocorticotropin release by enriched populations of corticotropes: Counteractions by arginine vasopressin and its second messengers. *Endocrinology* (1990) **126**: 1967-1975.

Chowdhury, G. M. I., Fujioka, T., and Nakamura, S. Induction and adaptation of Fos expression in the rat brain by two types of acute restraint stress. *Brain Research Bulletin* (2000) **52**: 171-182.

Chronwall, B. M., DiMaggio, D. A., Massari, V. J. and others. The anatomy of neuropeptide-Y-containing neurons in rat brain. *Neuroscience* (1985) **15**: 1159-1181.

Clarke, A. S. and Schneider, M. L. Prenatal stress has long-term effects on behavioral responses to stress in juvenile rhesus monkeys. *Developmental Psychobiology* (1993) **26**: 293-304.

Clarke, G., Wood, P., Merrick, L., and Lincoln, D. W. Opiate inhibition of peptide release from the neurohumoral terminals of hypothalamic neurones. *Nature* (1979) **282**: 746-748.

Claustre, Y., Rivy, J. P., Dennis, T., and Scatton, B. Pharmacological studies on stress-induced increase in frontal cortical dopamine metabolism in the rat. *Journal of Pharmacology & Experimental Therapeutics* (1986) **238**: 693-700.

Clements, A. D. The incidence of attention-deficit-hyperactivity disorder in children whose mothers experienced extreme psychological stress. *Georgia Educational Researcher* (1992) **91**: 1-14.

Clifton, V. L., Owens, P. C., Robinson, P. J., and Smith, R. Identification and characterization of a corticotrophin-releasing hormone receptor in human placenta *European Journal of Endocrinology* (1995) **133**: 591-597.

Colotta, F., Re, F., Muzio, M., Bertini, R., Polentarutti, N., Sironi, M., Giri, J. G., Dower, S. K., Sims, J. E., and Mantovani, A. Interleukin-1 type II receptor: A decoy target for IL-1 that is regulated by IL-4. *Science* (1993) **261**: 472-475.

Concas, A., Mostallino, M. C., Porcu, P., Follesa, P., Barbaccia, M. L., Trabucchi, M., Purdy, R. H., Grisenti, P., and Biggio, G. Role of brain allopregnanolone in the plasticity of gamma-aminobutyric acid type A receptor in rat brain during pregnancy and after delivery. *Proceedings of the National Academy of Sciences of the United States of America* (1998) **95**: 13284-13289.

Condon, J., Ricketts, M. L., Whorwood, C. B., and Stewart, P. M. Ontogeny and sexual dimorphic expression of mouse type 2 11beta-hydroxysteroid dehydrogenase. *Molecular & Cellular Endocrinology* (1997) **127**: 121-128.

Considine, R. V., Sinha, M. K., Heiman, M. L., Kriauciunas, A., Stephens, T. W., Nyce, M. R., Ohannesian, J. P., Marco, C. C., McKee, L. J., Bauer, T. L., and Caro, J. F. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *New England Journal of Medicine* (1996) **334**: 292-295.

Coons, A. H., Creech, H. L., and Jones, R. N. Immunological properties of an antibody containing a fluorescent group. *Proceedings of the Society for Experimental Biology and Medicine* (1941) **47**: 200-202.

Corpechot, C., Young, J., Calvel, M., Wehrey, C., Veltz, J. N., Touyer, G., Mouren, M., Prasad, V. V. K., Banner, C., Sjovall, J., Baulieu, E. E., and Robel, P. Neurosteroids: 3alpha-Hydroxy-5alpha-pregnan-20-one and its precursors in the brain, plasma, and steroidogenic glands of male and female rats. *Endocrinology* (1993) **133**: 1003-1009.

Cover, P. O. and Buckingham, J. C. Effects of selective opioid-receptor blockade on the hypothalamo-pituitary-adrenocortical responses to surgical trauma in the rat. *Journal of Endocrinology* (1989) **121**: 213-220.

Cowell, A. M. and Buckingham, J. C. Eicosanoids and the hypothalamo-pituitary axis. *Prostaglandins Leukotrienes & Essential Fatty Acids* (1989) **36**: 235-250.

Cratty, M. S., Ward, H. E., Johnson, E. A., Azzaro, A. J., and Birkle, D. L. Prenatal stress increases corticotropin-releasing factor (CRF) content and release in rat amygdala minces. *Brain Research* (1995) **675**: 297-302.

Crawford, P. A., Sadovsky, Y., Woodson, K., Lee, S. L., and Milbrandt, J. Adrenocortical function and regulation of the steroid 21-hydroxylase gene in NGFI-B-deficient mice. *Molecular & Cellular Biology* (1995) **15**: 4331-4336.

Cripps, A. W. and Williams, V. The effect of pregnancy and lactation on food intake, gastrointestinal anatomy and the absorptive capacity of the small intestine in the albino rat. *British Journal of Nutrition* (1975) **33**: 17-32.

Csapo, A. I. and Wiest, W. G. An examination of the quantitative relationship between progesterone and the maintenance of pregnancy. *Endocrinology* (1969) **85**: 735-746.

Cullinan, W. E., Helmreich, D. L., and Watson, S. J. Fos expression in forebrain afferents to the hypothalamic paraventricular nucleus following swim stress. *Journal of Comparative Neurology* (1996) **368**: 88-99.

Cullinan, W. E., Herman, J. P., Battaglia, D. F., Akil, H., and Watson, S. J. Pattern and time course of immediate early gene expression in rat brain following acute stress . *Neuroscience* (1995) **64**: 477-505.

Cullinan, W. E., Herman, J. P., and Watson, S. J. Ventral subicular interaction with the hypothalamic paraventricular nucleus: Evidence for a relay in the bed nucleus of the stria terminalis. *Journal of Comparative Neurology* (1993) **332**: 1-20.

Cummings, S., Elde, R., Ells, J., and Lindall, A. Corticotropin-releasing factor immunoreactivity is widely distributed within the central nervous system of the rat: An immunohistochemical study. *Journal of Neuroscience* (1983) **3**: 1355-1368.

Cunningham, E. T. Jr., Bohn, M. C., and Sawchenko, P. E. Organisation of the noradrenergic inputs to the paraventricular and supraoptic nuclei of the hypothalamus in the rat. *Journal of Comparative Neurology* (1990) **292**: 651-667.

Cunningham, E. T. Jr. and Sawchenko, P. E. Anatomical specificity of noradrenergic inputs to the paraventricular nucleus and supraoptic nucleus of the rat hypothalamus. *Journal of Comparative Neurology* (1988) **274**: 60-76.

Cunningham Jr, E. T. and Sawchenko, P. E. Reflex control of magnocellular vasopressin and oxytocin secretion. *Trends in Neurosciences* (1991) **14**: 406-411.

Cunningham Jr, E. T., Wada, E., Carter, D. B., Tracey, D. E., Battey, J. F., and De Souza, E. B. In situ histochemical localization of type I interleukin-1 receptor messenger RNA in the central nervous system, pituitary, and adrenal gland of the mouse. *Journal of Neuroscience* (1992) **12**: 1101-1114.

Cutler, D. J., Morris, R., Sheridhar, V., Wattam, T. A. K., Holmes, S., Patel, S., Arch, J. R. S., Wilson, S., Buckingham, R. E., Evans, M. L., Leslie, R. A., and Williams, G. Differential distribution of orexin-A and orexin-B immunoreactivity in the rat brain and spinal cord. *Peptides* (1999) **20**: 1455-1470.

Da Costa, A. P. C., Wood, S, Ingram, C. D., and Lightman, S. L. Region-specific reduction in stress-induced c-fos mRNA expression during pregnancy and lactation. *Brain Research* (1996) **742**: 177-184.

Da Costa, A. P. C., Kampa, R. J., Windle, R. J., Ingram, C. D., and Lightman, S. L. Region-specific immediate-early gene expression following the administration of corticotropin-releasing hormone in virgin and lactating rats. *Brain Research* (1997) **770**: 151-162.

Daikoku, S., Okamura, Y., Kawano, H. and others. Immunohistochemical study on the development of CRF-containing neurons in the hypothalamus of the rat. *Cell & Tissue Research* (1984) **238**: 539-544.

Dallman, M. F., Akana, S. F., Levin, N., Walker, C. D., Bradbury, M. J., Suemaru, S., and Scribner, K. S. Corticosteroids and the control of function in, the hypothalamo-pituitary-adrenal (HPA) axis. *Annals of the New York Academy of Sciences.* (1994) **746**: 22-32.

Dallman, M. F., Jones, M. T., Vernikos-Danellis, J., and Ganong, W. F. Corticosteroid feedback control of ACTH secretion: rapid effects of bilateral adrenalectomy on plasma ACTH in the rat. *Endocrinology* (1972) **91**: 961-968.

Dallman, M. F., Akana, S. F., Cascio, C. S., Darlington, D. N., Jacobson, L., and Levin, N. Regulation of ACTH secretion: variations on a theme of B. *Recent Progress in Hormone Research* (1987) **42**: 113-167.

Dallman, M. F., Engeland, W. C., Rose, J. C., Wilkinson, C. W., Shinsako, J., and Siedenburg, F. Nycthemeral rhythm in adrenal responsiveness to ACTH. *American Journal of Physiology* (1978) **235**: R210-R218.

Dallman, M. F., Levin, N., Cascio, C. S., Akana, S. F., Jacobson, L., and Kuhn, R. W. Pharmacological evidence that the inhibition of diurnal adrenocorticotropin secretion by corticosteroids is mediated via type I corticosterone-preferring receptors. *Endocrinology* (1989) **124**: 2844-2850.

Dallman, M. F. and Yates, F. E. Dynamic asymmetries in the corticosteroid feedback path and distribution-metabolism-binding elements of the adrenocortical system. *Annals of the New York Academy of Sciences* (1969) **156**: 696-721.

Darling, G., Goldstein, D. S., Stull, R., Gorschboth, C. M., and Norton, J. A. Tumor necrosis factor: Immune endocrine interaction. *Surgery* (1989) **106** : 1155-1160.

Date, Y., Ueta, Y., Yamashita, H., Yamaguchi, H., Matsukura, S., Kangawa, K., Sakurai, T., Yanagisawa, M., and Nakazato, M. Orexins, orexigenic hypothalamic peptides, interact with autonomic, neuroendocrine and neuroregulatory systems. *Proceedings of the National Academy of Sciences USA* (1999) **96**: 748-753.

Dautzenberg, F. M., Kilpatrick, G. J., Hauger, R. L., and Moreau, J. L. Molecular biology of the CRH receptors - In the mood. *Peptides* (2001) **22**: 753-760.

Davis, I. J., Hazel, T. G., and Lau, L. F. Transcriptional activation by Nur77, a growth factor-inducible member of the steroid hormone receptor superfamily. *Molecular Endocrinology* (1991) **5**: 854-859.

Davis, I. J. and Lau, L. F. Endocrine and neurogenic regulation of the orphan nuclear receptors Nur77 and Nurr-1 in the adrenal glands. *Molecular & Cellular Biology* (1994) **14**: 3469-3483.

Davis, M. The role of the amygdala in fear and anxiety. *Annual Review of Neuroscience.* (1992) **15**: 353-375.

Day, H. E. W. and Akil, H. Differential pattern of c-fos mRNA in rat brain following central and systemic administration of interleukin-1-beta: Implications for mechanism of action. *Neuroendocrinology* (1996) **63**: 207-218.

Dayas, C. V., Buller, K. M., and Day, T. A. Neuroendocrine responses to an emotional stressor: Evidence for involvement of the medial but not the central amygdala. *European Journal of Neuroscience* (1999) **11**: 2312-2322.

Dayas, C. V., Buller, K. M. Buller, Crane, J. W., Xu. Y, and Day, T. A. Stressor categorisation: acute physical and psychological stressors elicit distincive recruitment patterns in the amydala and in medullary noradrenergic cell groups. *European Journal of Neuroscience* (2001) **14**: 1143-1152.

De Goeij, D. C. E., Jezova, D., and Tilders, F. J. H. Repeated stress enhances vasopressin synthesis in corticotropin releasing factor neurons in the paraventricular nucleus. *Brain Research* (1992) **577**: 165-168.

De Goeij, D. C. E., Kvetnansky, R., Whitnall, M. H., Jezova, D., Berkenbosch, F., and Tilders, F. J. H. Repeated stress-induced activation of corticotropin-releasing factor neurons enhances vasopressin stores and colocalization with corticotropin-releasing factor in the median eminence of rats. *Neuroendocrinology* (1991) **53**: 150-159.

De Kloet, E. R., Ratka, A., Reul, J. M. H. M., Sutanto, W., and Van Eekelen, J. A. M. Corticosteroid receptor types in brain: Regulation and putative function. *Annals of the New York Academy of Sciences* (1987) **512**: 351-361.

De Kloet, E. R. and Reul, J. M. H. M. Feedback action and tonic influence of corticosteroids on brain function: A concept arising from the heterogeneity of brain receptor systems. *Psychoneuroendocrinology* (1987) **12**: 83-105.

De Kloet, E. R., Vreugdenhil, E., Oitzl, M. S., and Joels, M. Brain corticosteroid receptor balance in health and disease. *Endocrine Reviews* (1998) **19** : 269-301.

DeFrance, J. F. (ed) *In The Septal Nuclei* (1976)

De Lecea, L., Kilduff, T. S., Peyron, C., Gao, X. B., Foye, P. E., Danielson, P. E., Fukuhara, C., Battenberg, E. L. F., Gautvik, V. T., Bartlett, I. I. FS, Frankel, W. N., Van Den Pol, A. N., Bloom, F. E., Gautvik, K. M., and Sutcliffe, J. G. The hypocretins: Hypothalamus-specific peptides with neuroexcitatory activity. *Proceedings of the National Academy of Sciences of the United States of America* (1998) **95**: 322-327.

Delrue-Perollet, C., Li, K. S., Vitiello, S., and Neveu, P. J. Peripheral catecholamines are involved in the neuroendocrine and immune effects of LPS. *Brain, Behavior, & Immunity* (1995) **9**: 149-162.

Deminiere, J. M., Piazza, P. V., Guegan, G., Abrous, N., Maccari, S., Le Moal, M., and Simon, H. Increased locomotor response to novelty and propensity to intravenous amphetamine self-administration in adult offspring of stressed mothers. *Brain Research* (1992) **586**: 135-139.

Denton, D. A., McKinley, M. J., and Weisinger, R. S. Hypothalamic integration of body fluid regulation. *Proceedings of the National Academy of Sciences of the United States of America* (1996) **93**: 7397-7404.

De Souza, E. B. Corticotropin-releasing factor receptors in the rat CNS: Characterisation and regional distribution. *Journal of Neuroscience* (1987) **7**: 88-100.

DeSouza, E. B. and Kuhar, M. J. CRF receptors in the pituitary gland and CNS: Methods and overview. *Methods in Enzymology* (1986) **124**: 560-590.

DeWied, D. Melanotropins as Neuropeptides. *Annals of the New York Academy of Sciences* (1993) **680**: 20-28.

Diamant, M., Croiset, G., and De Wied, D. The effect of corticotropin-releasing factor (CRF) on autonomic and behavioral responses during shock-prod burying test in rats. *Peptides* (1992) **13**: 1149-1158.

Dijkstra, I., Tilders, F. J. H., Aguilera, G., Kiss, A., Rabadan-Diehl, C., Barden, N., Karanth, S., Holsboer, F., and Reul, J.M.H.M. Reduced activity of hypothalamic corticotropin-releasing hormone neurons in transgenic mice with impaired glucocorticoid receptor function. *Journal of Neuroscience* (1998) **18**: 3909-3918.

Dinarello, C. A. Interleukin-1. *Annals of the New York Academy of Sciences* (1988) **546**: 122-132.

Dohanics, J., Hoffman, G. E., and Verbalis, J. G. Hyponatremia-induced inhibition of magnocellular neurons causes stressor-selective impairment of stimulated adrenocorticotropin secretion in rats. *Endocrinology* (1991) **128**: 331-340.

Douglas, A. J., Dye, S., Leng, G., Russell, J. A., and Bicknell, R. J. Endogenous opioid regulation of oxytocin secretion through pregnancy in the rat. *Journal of Neuroendocrinology* (1993) **5**: 307-314.

Douglas, A. J., Johnstone, H., Brunton, P., and Russell, J. A. Sex-steroid induction of endogenous opioid inhibition on oxytocin secretory responses to stress. *Journal of Neuroendocrinology* (2000) **12**: 343-50.

Douglas, A. J., Johnstone, H., Hatzinger, M., Neumann, I., Landgraf, R., and Russell, J. A. Pregnancy reduced hypothalamo-pituitary-adrenal secretory responses to stressors in the rat. *Journal of Physiology* (1996) **495P**: 109P.

Douglas, A. J., Johnstone, H. A., Wigger, A, Landgraf, R, and Neumann, I. D. The role of endogenous opioids in neurohypophysial and hypothalamo-pituitary-adrenal axis hormone secretory responses to stress in pregnant rats. *Journal of Endocrinology* (1998) **158**: 285-293.

Douglas, A. J., Neumann, I., Meeren, H. K. M., Leng, G., Johnstone, L. E., Munro, G., and Russell, J. A. Central endogenous opioid inhibition of supraoptic oxytocin neurons in pregnant rats. *Journal of Neuroscience* (1995) **15**: 5049-5057.

Douglas, A. J., Scullion, S., Antonijevic, I. A., Brown, D., Russell, J. A., and Leng, G. Uterine contractile activity stimulates supraoptic neurons in term pregnant rats via a noradrenergic pathway. *Endocrinology* (2001) **142**: 633-644.

Drolet, G., Dumont E.C., Gosselin, I., Kinkead, R., Laforest, S., and Trottier, J. F. Role of endogenous opioid system in the regulation of the stress response. *Progress in Neuro-Psychopharmacology & Biological Psychiatry* (2001) **25**: 729-741.

Drouin, J., Charron, J., Gagner, J. P., Jeannotte, L., Nemer, M., Plante, R. K., and Wrange, O. Pro-opiomelanocortin gene: a model for negative regulation of transcription by glucocorticoids. *Journal of Cell Biochemistry* (1987) **35**: 293-304.

Drouin, J., Maira, M., and Philips, A. Novel mechanism of action for Nur77 and antagonism by glucocorticoids: A convergent mechanism for CRH activation and glucocorticoid repression of POMC gene transcription. *Journal of Steroid Biochemistry & Molecular Biology* (1998) **65**: 59-63.

Dunn, A. J., Berridge, C. W., Lai, Y. I., Yachabach, T. L., and File, S. E. Excessive grooming behavior in rats and mice induced by corticotropin-releasing factor. *Annals of the New York Academy of Sciences* (1988) **525**: 391-393.

Dunn, A. J. and Swiergiel, A. H. Behavioral responses to stress are intact in CRF-deficient mice. *Brain Research* (1999) **845**: 14-20.

Dunn, A. J. The role of interleukin-1 and tumor necrosis factor alpha in the neurochemical and neuroendocrine responses to endotoxin. *Brain Research Bulletin* (1992) **29**: 807-812.

Dunn, A. J. and Berridge, C. W. Physiological and behavioral responses to corticotropin-releasing factor administration: Is CRF a mediator of anxiety or stress responses? *Brain Research Reviews* (1990a) **15**: 71-100.

Dunn, A. J. and File, S. E. Corticotropin-releasing factor has an anxiogenic action in the social interaction test. *Hormones & Behavior* (1987) **21**: 193-202.

Dunn, J. and Orr, S. Differential plasma corticosterone responses to hippocampal stimulation. *Experimental Brain Research* (1984) **54**: 1-6.

Dunn, J. D. Plasma corticosterone responses to electrical stimulation of the bed nucleus of the stria terminalis. *Brain Research* (1987) **407**: 327-331.

Dunn, J. D. and Critchlow, V. Electrically stimulated ACTH release in pharmacologically blocked rats. *Endocrinology* (1973a) **93**: 835-842.

Dunn, J. D. and Critchlow, V. Pituitary-adrenal function following ablation of the medial basal hypothalamus. *Proceedings of the Society for Experimental Biology and Medicine* (1973b) **142**: 749-54.

Dunn, J. D. and Whitener, J. Plasma corticosterone responses to electrical stimulation of the amygdaloid complex: Cytoarchitectural specificity. *Neuroendocrinology* (1986) **42**: 211-217.

Dupouy, J. P., Coffigny, H., and Magre, S. Maternal and foetal corticosterone levels during late pregnancy in rats. *Journal of Endocrinology* (1975) **65**: 347-352.

DuVigneaud, V., Lawler, H. C., and Popenoe, E. A. Enzymatic cleavage of glycinamide from vasopressin and a proposed structure for this pressor-anti-diuretic hormone of the posterior piutitary. *Journal of the American Chemistry Society* (1953) **75**: 4880-4881.

Eberwine, J. H. and Roberts, J. L. Glucocorticoid regulation of pro-opiomelanocortin gene transcription in the rat pituitary. *Journal of Biological Chemistry* (1984) **259**: 2166-2170.

Edwards, C. R. W., Benediktsson, R., Lindsay, R. S., and Seckl, J. R. Dysfunction of placental glucocorticoid barrier: Link between fetal environment and adult hypertension? *Lancet* (1993) **341**: 355-357.

Edwards, C. M. B., Abusnana, S., Sunter, D., Murphy, K. G., Ghatei, M. A., and Bloom, S. R. The effect of the orexins on food intake: Comparison with neuropeptide Y, melanin-concentrating hormone and galanin. *Journal of Endocrinology* (1999) **160**: R7-R12.

Ehrhart-Bornstein, M., Hinson, J. P., Bornstein, S. R., Scherbaum, W. A., and Vinson, G. P. Intraadrenal interactions in the regulation of adrenocortical steroidogenesis. *Endocrine Reviews* (1998) **19**: 101-143.

Ek, M., Kurosawa, M., Lundeberg, T., and Ericsson, A. Activation of vagal afferents after intravenous injection of interleukin-1beta: Role of endogenous prostaglandins. *Journal of Neuroscience* (1998) **18**: 9471-9479.

Elias, C. F., Lee, C., Kelly, J., Aschkenasi, C., Ahima, R. S., Couceyro, P. R., Kuhar, M. J., Saper, C. B., and Elmquist, J. K. Leptin activates hypothalamic CART neurons projecting to the spinal cord. *Neuron* (1998) **21**: 1375-85.

Elmquist, J. K., Elias, C. F., and Saper, C. B. From lesions to leptin: Hypothalamic control of food intake and body weight. *Neuron* (1999) **22**: 221-232.

Emeson, R. B. and Eipper, B. A. Characterization of pro-ACTH/endorphin-derived peptides in rat hypothalamus. *Journal of Neuroscience* (1986) **6**: 837-849.

Engeland, W. C., Shinsako, J., and Dallman, M. F. Corticosteroids and ACTH are not required for compensatory adrenal growth. *American Journal of Physiology* (1975) **229**: 1461-1464.

Ericsson, A., Arias, C., and Sawchenko, P. E. Evidence for an intramedullary prostaglandin-dependent mechanism in the activation of stress-related neuroendocrine circuitry by intravenous interleukin-1. *Journal of Neuroscience* (1997) **17**: 7166-7179.

Ericsson, A., Kovacs, K. J., and Sawchenko, P. E. A functional anatomical analysis of central pathways subserving the effects of interleukin-1 on stress-related neuroendocrine neurons. *Journal of Neuroscience* (1994) **14**: 897-913.

Ericsson, A., Liu, C., Hart, R. P., and Sawchenko, P. E. Type 1 interleukin-1 receptor in the rat brain: Distribution, regulation, and relationship to sites of IL-1-induced cellular activation. *Journal of Comparative Neurology* (1995) **361**: 681-698.

Evans, R. M. and Arriza, J. L. A molecular framework for the actions of glucocorticoid hormones in the nervous system. *Neuron* (1989) **2**: 1105-1112.

Farrar, W. L., Kilian, P. L., Ruff, M. R. and others Visualization and characterization of interleukin 1 receptors in brain. *Journal of Immunology* (1987) **139**: 459-463.

Feldman, S., Conforti, N., Itzik, A., and Weidenfeld, J. Differential effect of amygdaloid lesions on CRF-41, ACTH and corticosterone responses following neural stimuli. *Brain Research* (1994) **658**: 21-26.

Fenelon, V. S. and Herbison, A. E. Plasticity in GABA(A) receptor subunit mRNA expression by hypothalamic magnocellular neurons in the adult rat. *Journal of Neuroscience* (1996) **16**: 4872-4880.

Fenelon, V. S. and Herbison, A. E. Progesterone regulation of GABA(A) receptor plasticity in adult rat supraoptic nucleus. *European Journal of Neuroscience* (2000) **12**: 1617-1623.

Fisher, L. A. and Brown, M. R. Central regulation of stress responses: Regulation of the autonomic nervous system and visceral function by corticotrophin releasing factor-41. *Baillieres Clinical Endocrinology & Metabolism* (1991) **5** : 35-50.

Flannelly, K. and Lore, R. The influence of females upon aggression in domesticates male rats (Rattus norvegicus). *Animal Behaviour* (1977) **25**: 654-659.

Fleischer, N. and Rawls, W. E. ACTH synthesis and release in pituitary monolayer culture: effect of dexamethasone. *American Journal of Physiology* (1970) **219**: 445-448.

Fleshner, M., Goehler, L. E., Hermann, J., Relton, J. K., Maier, S. F., and Watkins, L. R. Interleukin-1beta induced corticosterone elevation and hypothalamic NE depletion is vagally mediated. *Brain Research Bulletin* (1995) **37**: 605-610.

Fleshner, M., Deak, T., Spencer, R. L., Laudenslager, M. L., Watkins, L. R., and Maier, S. F. A long term increase in basal levels of corticosterone and a decrease in corticosteroid-binding globulin after acute stressor exposure. *Endocrinology* (1995) **136**: 5336-5342.

Fleshner, M., Silbert, L., Deak, T., Goehler, L. E., Martin, D., Watkins, L. R., and Maier, S. F. TNF-alpha-induced corticosterone elevation but not serum protein or corticosteroid binding globulin reduction is vagally mediated. *Brain Research Bulletin* (1997) **44**: 701-706.

Fortier C. Dual control of adrenocorticotropin release. *Endocrinology* (1951) **49**: 782-788.

Foxwell, B. M. J., Barrett, K., and Feldmann, M. Cytokine receptors: Structure and signal transduction. *Clinical & Experimental Immunology* (1992) **90**: 161-169.

Fride, E., Dan, Y., Feldon, J. and others. Effects of prenatal stress on vulnerability to stress in prepubertal and adult rats. *Physiology & Behavior* (1986) **37**: 681-687.

Fride, E. and Weinstock, M. The effects of prenatal exposure to predictable or unpredictable stress on early development in the rat. *Developmental Psychobiology* (1984) **17**: 651-660.

Fride, E. and Weinstock, M. Prenatal stress increases anxiety related behavior and alters cerebral lateralization of dopamine activity. *Life Sciences* (1988) **42**: 1059-1065.

Fukata, J., Usui, T., Naitoh, Y., Nakai, Y., and Imura, H. Effects of recombinant human interleukin-1alpha, -1beta, 2 and 6 on ACTH synthesis and release in the mouse pituitary tumour cell line AtT-20. *Journal of Endocrinology* (1989) **122**: 33-39.

Gall, G. and Pardue, M. L. Formation and detection of RNA-DNA hybrid molecules in cytological preparations. *Proceedings of the National Academy of Science USA* (1969) **63**: 378-383.

Gand D.S. *Annals of the New York Academy of Sciences* (1977) **297**: 477-496.

Ganten, D., Unger, T. H., and Lang, R. E. The dual role of angiotensin and vasopressin as plasma hormones and neuropeptides in cardiovascular regulation. *Journal de Pharmacologie* (1985) **16**: 51-68.

Garcia-Garcia, L, Harbuz, M. S., Manzanares, J, Lightman, S. L., and Fuentes, J. A. RU-486 blocks stress-induced enhancement of proenkephalin gene expression in the paraventricular nucleus of rat hypothalamus *Brain Research* (1998) **786**: 215-218.

Gatti, S. and Bartfai, T. Induction of tumor necrosis factor-alpha mRNA in the brain after peripheral endotoxin treatment: comparison with interleukin-1 family and interleukin-6. *Brain Research 1993* (1993) **624**: 291-4.

Gaykema, R. P. A., Dijkstra, I., and Tilders, F. J. H. Subdiaphragmatic vagotomy suppresses endotoxin-induced activation of hypothalamic corticotropin-releasing hormone neurons and ACTH secretion. *Endocrinology* (1995) **136**: 4717-4720.

Geenen, V., Robert, F., Defresne, M. P., Boniver, J., Legros, J. J., and Franchimont, P. Neuroendocrinology of the thymus. *Hormone Research* (1989) **31**: 81-84.

Gibbs, D. M. High concentrations of oxytocin in hypophysial portal plasma. *Endocrinology* (1984) **114**: 1216-1218.

Gibbs, D. M. Immunoneutralization of oxytocin attenuates stress-induced corticotropin secretion in the rat. *Regulatory Peptides* (1985) **12**: 273-277.

Gibbs, D. M. Stress-specific modulation of ACTH secretion by oxytocin *Neuroendocrinology* (1986a) **42**: 456-458.

Gibbs, D. M. Vasopressin And Oxytocin - Hypothalamic Modulators Of The Stress Response - A Review. *Psychoneuroendocrinology* (1986b) **11**: 131-140.

Gibbs, D. M. and Vale, W. Presence of corticotropin releasing factor-like immunoreactivity in hypophysial portal blood. *Endocrinology* (1982) **111**: 1418-1420.

Gillies, G. E., Linton, E. A., and Lowry, P. J. Corticotropin releasing activity of the new CRF is potentiated several times by vasopressin. *Nature* (1982) **299**: 355-357.

Gillis, S. Cytokine receptors. *Current Opinion in Immunology* (1991) **3**: 315-319.

Givalois, L., Gaillet, S., Mekaouche, M., Ixart, G., Bristow, A. F., Siaud, P., Szafarczyk, A., Malaval, F., Assenmacher, I., and Barbanel, G. Deletion of the ventral noradrenergic bundle obliterates the early ACTH response after systemic LPS, independently from the plasma IL-1beta surge. *Endocrine* (1995) **3** : 481-485.

Givalois, L., Siaud, P., Mekaouche, M., Ixart, G., Malaval, F., Assenmacher, I., and Barbanel, G. Early hypothalamic activation of combined Fos and CRH41 immunoreactivity and of CRH41 release in push-pull cannulated rats after systemic endotoxin challenge. *Molecular & Chemical Neuropathology* (1995) **26**: 171-186.

Goldsmith, L. T., Weiss, G., and Steinetz, B. G. Relaxin and its role in pregnancy. *Endocrinology and Metabolism Clinics of North America* (1995) **24**: 171-86.

Gomez, F, Lahmame, A, deKloet, E. R., and Armario, A. Hypothalamic-Pituitary-Adrenal Response To Chronic Stress In Five Inbred Rat Strains: Differential Responses Are Mainly Located At The Adrenocortical Level. *Neuroendocrinology* (1996) **63**: 327-337.

Gray, T. S., Piechowski, R. A., Yracheta, J. M., Rittenhouse, P. A., Bethea, C. L., and Van de Kar, L. D. Ibotenic acid lesions in the bed nucleus of the Stria terminalis attenuate condition stress-induced increases in prolactin, ACTH and corticosterone. *Neuroendocrinology* (1993) **57**: 517-524.

Grigoriadis, D. E. and De Souza, E. B. Corticotropin-releasing factor (CRF) receptors in intermediate lobe of the pituitary: Biochemical characterization and autoradiographic localization. *Peptides* (1989) **10**: 179-188.

Grigoriadis, D. E., Lovenberg, T. W., Chalmers, D. T., Liaw, C., and De Souza, E. B. Characterization of corticotropin-releasing factor receptor subtypes. *Annals of the New York Academy of Sciences.* (1996) **780**: 60-80.

Guillaume, V., Conte-Devolx, B., Szafarczyk, A. and others. The corticotropin-releasing factor release in rat hypophysial portal blood is mediated by brain catecholamines. *Neuroendocrinology* (1987) **46**: 143-146.

Guillemin, R. and Rosenberg, B. Humoral hypothalamic control of anterior pituitary: A study with combined tissue cultures. *Endocrinology* (1955) **57**: 599-607.

Gunnet, J. and Freeman, M. The mating-induced release of prolactin: a unique neuroendocrine response. *Endocrine Reviews* (1983) **4**: 44-61.

Gurdjian, E. S. The diencephalon of the albino rat. *Journal of Comparative Neurology* (1927) **43**: 1-114.

Gutman, D. A., Owens, M. J., and Nemeroff, C. B. CRF receptor antagonists: A new approach to the treatment of depression. *Pharmaceutical News* (2001) **8** : 18-25.

Gwosdow, A. R., O'Connell, N. A., Spencer, J. A., Kumar, M. S. A., Agarwal, R. K., Bode, H. H., and Abou-Samra, A. B. Interleukin-1-induced corticosterone release occurs by an adrenergic mechanism from rat adrenal gland. *American Journal of Physiology - Endocrinology & Metabolism* (1992) **263**: E461-E466.

Habu, S., Watanobe, H., Yasujima, M., and Suda, T. Different roles of brain interleukin 1 in the adrenocorticotropin response to central versus peripheral administration of lipopolysaccharide in the rat. *Cytokine* (1998) **10**: 390-394.

Hagan, P., Poole, S., and Bristow, A. F. Endotoxin-stimulated production of rat hypothalamic interleukin-1beta in vivo and in vitro, measured by specific immunoradiometric assay. *Journal of Molecular Endocrinology* (1993) **11**: 31-36.

Hagan, J. J., Leslie, R. A., Patel, S., Evans, M. L., Wattam, T. A., Holmes, S., Benham, C. D., Taylor, S. G., Routledge, C., Hemmati, P., Munton, R. P., Ashmeade, T. E., Shah, A. S., Hatcher, J. P., Hatcher, P. D., Jones, D. N. C., Smith, M. I., Piper, D. C., Hunter, A. J., Porter, R. A., and Upton, N. Orexin A activates locus coeruleus cell firing and increases arousal in the rat. *Proceedings of the National Academy of Sciences of the United States of America* (1999) **96**: 10911-10916.

Hagiwara, M., Brindle, P., Harootunian, A., Armstrong, R., Rivier, J., Vale, W., Tsien, R., and Montminy MR. Coupling of hormonal stimulation and transcription via the cyclic AMP-responsive factor CREB is rate limited by nuclear entry of protein kinase A. *Moleuclar Cell Biology* (1993) **13**: 4852-4859.

Hahn, T. M., Breininger, J. F., Baskin, D. G., and Schwartz, M. W. Coexpression of Agrp and NPY in fasting-activated hypothalamic neurons. *Nature Neuroscience* (1998) **1**: 271-272.

Haller, J., Barna, I., and Baranyi, M. Hormonal and metabolic responses during psychosocial stimulation in aggressive and nonaggressive rats. *Psychoneuroendocrinology* (1995) **20**: 65-74.

Hammond, G. L. Molecular properties of corticosteroid binding globulin and the sex-steroid binding proteins. *Endocrine Reviews* (1990) **11**: 65-79.

Hammond, G. L., Smith, C. L., Goping, I. S. and others. Primary structure of human corticosteroid binding globulin, deduced from hepatic and pulmonary cDNAs, exhibits homology with serine protease inhibitors *Proceedings of the National Academy of Sciences of the United States of America* (1987) **84**: 5153-5157.

Hansen, M. K., Taishi, P., Chen, Z., and Krueger, J. M. Vagotomy blocks the induction of interleukin-1beta (IL-1beta) mRNA in the brain of rats in response to systemic IL-1beta. *Journal of Neuroscience* (1998) **18**: 2247-2253.

Hanson, E. S. and Dallman, M. F. Neuropeptide Y (NPY) may integrate responses of hypothalamic feeding systems and the hypothalamo-pituitary-adrenal axis. *Journal of Neuroendocrinology* (1995) **7**: 273-279.

Harbuz, M. S., Rees, R. G., Eckland, D., Jessop, D. S., Brewerton, D., and Lightman, S. L. Paradoxical responses of hypothalamic corticotropin-releasing factor (CRF) messenger ribonucleic acid (mRNA) and CRF-41 peptide and adenohypophysial proopiomelanocortin mRNA during chronic inflammatory stress. *Endocrinology* (1992) **130**: 1394-1400.

Harbuz, M. S., Jessop, D. S, Lightman, S. L., and Chowdrey, S. L. The Effects Of Restraint Or Hypertonic Saline Stress On Corticotropin-Releasing Factor, Arginine-Vasopressin, And Proenkephalin-A Messenger-RNAs Is The CFY, Sprague-Dawley And Wistar Strains Of Rat. *Brain Research* (1994) **667**: 6-12.

Harbuz, M., Russell, J. A., Sumner, B. E. H., Kawata, M., and Lightman, S. L. Rapid changes in the content of proenkephalin A and corticotrophin releasing hormone mRNAs in the paraventricular nucleus during morphine withdrawal in urethane-anaesthetized rats. *Molecular Brain Research* (1991) **9**: 285-291.

Harbuz, M. S. and Lightman, S. L. Responses of hypothalamic and pituitary mRNA to physical and psychological stress in the rat. *Journal of Endocrinology* (1989) **122**: 705-711.

Harbuz, M. S., Stephanou, A., Sarlis, N., and Lightman, S. L. The effects of recombinant human interleukin (IL)-1alpha, IL-1beta or IL-6 on hypothalamo-pituitary-adrenal axis activation. *Journal of Endocrinology* (1992b) **133**: 349-355.

Harlan, R. E., Shivers, B. D., Romano, G. J. and others Localization of preproenkephalin mRNA in the rat brain and spinal cord by in situ hybridization. *Journal of Comparative Neurology* (1987) **258**: 159-184.

Harris, G. W. Neural Control of the Pituitary Gland. *Physiological Reviews* (1948) **28**: 139-179.

Hashimoto, K., Suemaru, S., Takao, T., Sugawara, M., Makino, S., and Ota, Z. Corticotropin-releasing hormone and pituitary-adrenocortical responses in chronically stressed rats. *Regulatory Peptides* (1988) **23**: 117-126.

Hauger, R. L., Lorang, M., Irwin, M., and Aguilera, G. CRF receptor regulation and sensitization of ACTH responses to acute ether stress during chronic intermittent immobilization stress. *Brain Research* (1990) **532**: 34-40.

Hauger, R. L., Millan, M. A., Catt, K. J., and Aguilera, G. Differential regulation of brain and pituitary corticotropin-releasing factor receptors by corticosterone. *Endocrinology* (1987) **120**: 1527-1533.

Hayashi, A., Nagaoka, M., Yamada, K., Ichitani, Y., Miake, Y., and Okado, N. Maternal stress induces synaptic loss and developmental disabilities of offspring. *International Journal of Developmental Neuroscience* (1998) **16**: 209-216.

Hayes, A. G. and Stewart, B. R. Effect of mu and kappa opioid receptor agonists on rat plasma corticosterone levels. *European Journal of Pharmacology* (1985) **116**: 75-79.

Haynes, R. C. and Berthet, L. Studies on the mechanism of action of the adrenocorticotropic hormone. *Journal of Biological Chemistry* (1957) **225**: 115-124.

Haynes, A. C., Jackson, B., Overend, P., Buckingham, R. E., Wilson, S., Tadayyon, M., and Arch, J. R. S. Effects of single and chronic intracerebroventricular administration of the orexins on feeding in the rat. *Peptides* (1999) **20**: 1099-1105.

Hazel, T. G., Nathans, D., and Lau, L. F. A gene inducible by serum growth factors encodes a member of the steroid and thyroid hormone receptor superfamily. *Proceedings of the National Academy of Sciences of the United States of America* (1988) **85**: 8444-8448.

Heine, P. A., Di, S., Ross, L. R., Anderson, L. L., and Jacobson, C. D. Relaxin-induced expression of Fos in the forebrain of the late pregnant rat. *Neuroendocrinology* (1997) **66**: 38-46.

Heinrichs, S. C., Pich, E. M., Miczek, K. A., Britton, K. T., and Koob, G. F. Corticotropin-releasing factor antagonist reduces emotionality in socially defeated rats via direct neurotropic action. *Brain Research* (1992) **581**: 190-197.

Hench, P. S. and Kendall, E. C. Effects of cortisone acetate and pituitary ACTH on rheumatoid arthritis, rheumatic fever and certain other conditions. *Archives of International Medicine* (1950)

Henry, C., Kabbaj, M., Simon, H., Le Moal, M., and Maccari, S. Prenatal stress increases the hypothalamo-pituitary-adrenal axis response in young and adult rats *Journal of Neuroendocrinology* (1994) **6**: 341-345.

Herbert, E., Allen, R. G., and Paquette, T. L. Reversal of dexamethasone inhibition of adrenocorticotropin release in a mouse pituitary tumor cell line either by growing cells in the absence of dexamethasone or by addition of hypothalamic extract. *Endocrinology* (1978) **102**: 218-226.

Herman, J. P., Schafer MK-H, Young, E. A., Thompson, R., Douglass, J., Akil, H., and Watson, S. J. Evidence for hippocampal regulation of neuroendocrine neurons of the hypothalamo-pituitary-adrenocorticoid axis. *Journal of Neuroscience* (1989) **9**: 3072-3082.

Herman, J. P., Cullinan, W. E., Young, E. A., Akil, H., and Watson, S. J. Selective forebrain fiber tract lesions implicate ventral hippocampal structures in tonic regulation of paraventricular nucleus corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) mRNA expression. *Brain Research* (1992) **592**: 228-238.

Herman, J. P. and Cullinan, W. E. Neurocircuitry of stress: Central control of the hypothalamo-pituitary-adrenocortical axis. *Trends in Neurosciences* (1997) **20**: 78-84.

Herman, J. P., Cullinan, W. E., and Watson, S. J. Involvement of the bed nucleus of the stria terminalis in tonic regulation of paraventricular hypothalamic CRH and AVP mRNA expression. *Journal of Neuroendocrinology* (1994) **6**: 433-442.

Herman, J. P., Prewitt CM-F, and Cullinan, W. E. Neuronal circuit regulation of the hypothalamo-pituitary-adrenocortical stress axis. *Critical Reviews in Neurobiology* (1996) **10**: 371-394.

Higuchi, T., Bicknell, R. J., and Leng, G. Reduced oxytocin release from the neural lobe of lactating rats is associated with reduced pituitary content and does not reflect reduced excitability of oxytocin neurons. *Journal of Neuroendocrinology* (1991) **3**: 297-302.

Higuchi, T., Honda, K., Fukuoka, T. and others. Release of oxytocin during suckling and parturition in the rat. *Journal of Endocrinology* (1985) **105**: 339-346.

Higuchi, T., Honda, K., Takano, S., and Negoro, H. Reduced oxytocin response to osmotic stimulus and immobilization stress in lactating rats. *Journal of Endocrinology* (1988) **116**: 225-230.

Higuchi, T., Negoro, H., and Arita, J. Reduced responses of prolactin and catecholamine to stress in the lactating rat. *Journal of Endocrinology* (1989) **122**: 495-498.

Hillhouse, E. W. and Jones, M. T. Effect of bilateral adrenalectomy and corticosteroid therapy on the secretion of corticotrophin-releasing factor activity from the hypothalamus of the rat in vitro. *Journal of Endocrinology* (1976) **71**: 21-30.

Hisano, S., Tsuruo, Y., Katoh, S., Daikoku, S., Yanaihara, N., and Shibasaki, T. Intragranular colocalization of arginine vasopressin and methionine-enkephalin-octapeptide in CRF-axons in the rat median eminence. *Cell and Tissue Research* (1987) **249**: 497-507.

Hodgkinson, S. C., Allolio, B., Landon, J., and Lowry, P. J. Development of an non-extracted two-site immunoradiometric assay for corticotropin utilising extreme amino- and carboxy terminally directed antibodies. *Biochemical Journal* (1984) **218**: 703-711.

Hodges, J. R. and Sadow, J. Impairment of pituitary-adrenocorticotropic function by corticosterone in the blood. *British Journal of Pharmacology* (1967) **30**: 385.

Hoffman, G. E., Smith, M. S., and Verbalis, J. G. c-Fos and related immediate early gene products as markers of activity in neuroendocrine systems. *Frontiers in Neuroendocrinology* (1993) **14**: 173-213.

Hokfelt, T., Fahrenkrug, J., Tatemoto, K. and others The PHI (PHI-27)/corticotropin-releasing factor/enkephalin immunoreactive hypothalamic neuron: Possible morphological basis for integrated control of prolactin, corticotropin, and growth hormone secretion. *Proceedings of the National Academy of Sciences of the United States of America* (1983) **80**: 895-898.

Holmes, M. C., Antoni, F. A., Aguilera, G., and Catt, K. J. Magnocellular axons in passage through the median eminence release vasopressin. *Nature* (1986a) **319**: 326-329.

Holmes, M. C., Antoni, F. A., Catt, K. J., and Aguilera, G. Predominant release of vasopressin vs. Corticotropin-releasing factor from the isolated median eminence after adrenalectomy. *Neuroendocrinology* (1986b) **43**: 245-251.

Holmes, M. C., Catt, K. J., and Aguilera, G. Involvement of vasopressin in the down-regulation of pituitary corticotropin-releasing factor receptors after adrenalectomy. *Endocrinology* (1987) **121**: 2093-2098.

Holmes, M. C., Kotelevtsev, Y., Mullins, J. J., and Seckl, J. R. Phenotypic analysis of mice bearing targeted deletions of 11beta-hydroxysteroid dehydrogenases 1 and 2 genes. *Molecular & Cellular Endocrinology* (2001) **171**: 15-20.

Honkaniemi, J., Kononen, J., Kainu, T., Pyykonen, I., and Pelto-Huikko, M. Induction of multiple immediate early genes in rat hypothalamic paraventricular nucleus after stress. *Molecular Brain Research* (1994) **25**: 234-241.

Horvath, T. L., Diano, S., and Van den Pol, A. N. Synaptic interaction between hypocretin (orexin) and neuropeptide Y cells in the rodent and primate hypothalamus: A novel circuit implicated in metabolic and endocrine regulations. *Journal of Neuroscience* (1999) **19**: 1072-1087.

Hosoya, Y. and Matsushita M. Identification and distribution of the spinal and hypophysial projection neurones in the PVN of the rat. A light and electron microscopic study with the horseradish peroxidase method. *Experimental Brain Research* (1979) **35**: 315-331.

Huttunen, M. O. and Niskanen, P. Prenatal loss of father and psychiatric disorders. *Archives of General Psychiatry* (1978) **35**: 429-431.

Ida, T., Nakahara, K., Katayama, T., Murakami, N., and Nakazato, M. Effect of lateral cerebroventricular injection of the appetite- stimulating neuropeptide, orexin and neuropeptide Y, on the various behavioral activities of rats. *Brain Research* (1999) **821**: 526-529.

Ida, T., Nakahara, K., Murakami, T., Hanada, R., Nakazato, M., and Murakami, N. Possible involvement of orexin in the stress reaction in rats. *Biochemical and Biophysical Research Communications* (2000) **270**: 318-323.

Imaki, T., Nahan, J. L., Rivier, C., Sawchenko, P. E., and Vale, W. Differential regulation of corticotropin-releasing factor mRNA in rat brain regions by glucocorticoids and stress. *Journal of Neuroscience* (1991a) **11**: 585-599.

Imaki, T, Naruse, M, Harada, S, Chikada, N, Imaki, J, Onodera, H, Demura, H, and Vale, W Corticotropin-releasing factor up-regulates its own receptor mRNA in the paraventricular nucleus of the hypothalamus. *Molecular Brain Research* (1996a) **38**: 166-170.

Imaki, T, Shibasaki, T, Chikada, N, Harada, S, Naruse, M, and Demura, H Different expression of immediated early genes in the rat paraventricular nucleus induced by stress. Relation to corticotropin releaing factor gene transcription. *Endocrine Journal* (1996b) **43**: 629-638.

Imaki, T., Shibasaki, T., Hotta, M., and Demura, H. Intracerebroventricular administration of corticotropin-releasing factor induces c-fos mRNA expression in brain regions related to stress responses: Comparison with pattern of c-fos mRNA induction after stress. *Brain Research* (1993) **616**: 114-125.

Imaki, T, Wang, X. Q., Shibasaki, T, Yamada, K, Harada, S, Chikada, N, Naruse, M, and Demura, H Stress-induced activation of neuronal activity and corticotropin-releasing factor gene-expression in the paraventricular nucleus is modulated by glucocorticoids in rats. *Journal of Clinical Investigation* (1995) **96**: 231-238.

Insel, T. R., Kinsley, C. H., Mann, P. E., and Bridges, R. S. Prenatal stress had long-term effects on brain opiate receptors. *Brain Research* (1990) **511**: 93-97.

Ishizuka, T., Wei, X., and Kubo, T. Cardiovascular effects of microinjections of thyrotropin-releasing hormone, oxytocin and other neuropeptides into the rostral ventrolateral medulla of the rat. *Archives Internationales de Pharmacodynamie et de Therapie.* (1993) **322**: 35-44.

Ivell, R. and Richter, D. Structure and comparison of the oxytocin and vasopressin genes from rat. *Proceedings of the National Academy of Sciences of the United States of America* (1984) **81**: 2006-2010.

Jaszberenyi, M., Bujdoso, E., Pataki, I., and Telegdy, G. Effects of orexins on the hypothalamic-pituitary-adrenal system. *Journal of Neuroendocrinology* (2000) **12**: 1174-1178.

Jaszberenyi, M., Bujdoso, E., and Telegdy, G. The role of neuropeptide Y in orexin-induced hypothalamic-pituitary-adrenal activation. *Journal of Neuroendocrinology* (2001) **13**: 438-441.

Jeanrenaud, B. and Rohner-Jeanrenaud, F. Effects of neuropeptides and leptin on nutrient partitioning: Dysregulations in obesity. *Annual Review of Medicine* (2001) **52**: 339-351.

Jessop, D. S. Central non-glucocorticoid inhibitors of the hypothalamo-pituitary-adrenal axis. *Journal of Endocrinology* (1999) **160**: 169-180.

Jezova, D., Michajlovskij, N., Kvetnansky, R., and Makara, G. B. Paraventricular and supraoptic nuclei of the hypothalamus are not equally important for oxytocin release during stress. *Neuroendocrinology* (1993) **57**: 776-781.

Jia, H. G., Rao, Z. R., and Shi, J. W. Projection from the ventrolateral medullary neurons containing tyrosine hydroxylase to the central amygdaloid nucleus in the rat. *Brain Research* (1992) **589**: 167-170.

Jingami, H., Matsukura, S., Numa, S., and Imura, H. Effects of adrenalectomy and dexamethasone administration on the level of prepro-corticotropin-releasing factor messenger ribonucleic acid (mRNA) in the hypothalamus and adrenocorticotropin/beta-lipotropin precursor mRNA in the pituitary in rats. *Endocrinology* (1985a) **117**: 1314-20.

Jingami, H., Mizuno, N., Takahashi, H., Shibahara, S., Furutani, Y., Imura, H., and Numa, S. Cloning and sequence analysis of cDNA for rat corticotropin-releasing factor precursor. *FEBS Letters* (1985b) **191**: 63-6.

Johnstone, H. A., Wigger, A., Douglas, A. J., Neumann, I. D., Landgraf, R., Seckl, J. R., and Russell, J. A. Attenuation of hypothalamic-pituitary-adrenal axis stress responses in late pregnancy: Changes in feedforward and feedback mechanisms. *Journal of Neuroendocrinology* (2000a) **12**: 811-822.

Johnstone, L. E., Brown, C. H., Meeren, H. K. M., Vuijst, C. L., Brooks, P. J., Leng, G., and Russell, J. A. Local morphine withdrawal increases c-fos gene, fos protein, and oxytocin gene expression in hypothalamic magnocellular neurosecretory cells. *Journal of Neuroscience* (2000b) **20**: 1272-1280.

Johnstone, L. E. and Higuchi, T. Food intake and leptin during pregnancy and lactation. *Progress in Brain Research* (2001) **133**: 215-227.

Jones, C. T., Gu, W., and Parer, J. T. Production of corticotrophin releasing hormone by the sheep placenta in vivo *Journal of Developmental Physiology* (1989) **11**: 97-101.

Jones, E. G. and Hatman, B. K. Recent advances in neuroanatomical methodolgy *Annual Review of Neuroscience* (1978) **1**: 215-296.

Jones, M. T. and Hillhouse, E. W. Structure-activity relationship and the mode of action of corticosteroid feedback on the secretion of corticotrophin-releasing factor (corticoliberin). *Journal of Steroid Biochemistry* (1976) **7**: 1189-1202.

Jones, M. T., Hillhouse, E. W., and Burden, J. L. Dynamics and mechanics of corticosteroid feedback at the hypothalamus and anterior pituitary gland. *Journal of Endocrinology* (1977) **73**: 405-417.

Jones, M. T., Tiptaft, E. M., Brush, F. R., Fergusson, D. A., and Neame, R. L. Evidence for dual corticosteroid-receptor mechanisms in the feedback control of adrenocorticotrophin secretion. *Journal of Endocrinology* (1974) **60**: 223-233.

Joseph, S. A., Pilcher, W. H., and Bennett Clarke, C. Immunocytochemical localization of ACTH perikarya in nucleus tractus solitarius: Evidence for a second opiocortin neuronal system. *Neuroscience Letters* (1983) **38**: 221-225.

Kaada, B. R. *In The Neurobiology of the Amygdala (ed. B.E. Eleftheriou)* (1972) 205-281.

Kakouris, H., Eddie, L. W., and Summers, R. J. Relaxin: more than just a hormone of pregnancy. *Trends in Pharmacological Science* (1993) **14**: 4-6.

Kakucska, I., Qi, Y., Clark, B. D., and Lechan, R. M. Endotoxin-induced corticotropin-releasing hormone gene expression in the hypothalamic paraventricular nucleus is mediated centrally by interleukin-1. *Endocrinology* (1993) **133** : 815-821.

Kalin, N. H., Insel, T. R., Cohen, R. M. and others. Diurnal variation in cerebrospinal fluid prolactin concentration of the rhesus monkey. *Journal of Clinical Endocrinology & Metabolism* (1981) **52**: 857-858.

Kalra, S. P., Dube, M. G., Sahu, A., Phelps, C. P., and Kalra, P. S. Neuropeptide Y secretion increases in the paraventricular nucleus in association with increased appetite for food. *Proceedings of the National Academy of Sciences of the United States of America* (1991) **88**: 10931-10935.

Kaneko, M. and Hiroshige, T. Fast, rate-sensitive corticosteroid negative feedback during stress. *American Journal of Physiology* (1978) **234**: R39-R45.

Kaneko, M., Hiroshige, T., Shinsako, J., and Dallman, M. F. Diurnal changes in amplification of hormone rhythms in the adrenocortical system. *American Journal of Physiology - Regulatory Integrative & Comparative Physiology* (1980) **8**: R309-R316.

Kanosue, K., Hosono, T., Zhang, Y. H., and Chen, X. M. Neuronal networks controlling thermoregulatory effectors. *Progress in Brain Research* (1998) **115** : 49-62.

Kant, G. J., Eggleston, T., Landman-Roberts, L. and others. Habituation to repeated stress is stressor specific *Pharmacology, Biochemistry & Behavior* (1985) **22**: 631-634.

Kapas, L., Hansen, M. K., Chang, H. Y., and Krueger, J. M. Vagotomy attenuates but does not prevent the somnogenic and febrile effects of lipopolysaccharide in rats *American Journal of Physiology - Regulatory Integrative & Comparative Physiology* (1998) **274**: R406-R411.

Karteris, E., Grammatopoulos, D., Dai, Y., Olah, K. B., Ghobara, T. B., Easton, A., and Hillhouse, E. W. The human placenta and fetal membranes express the corticotropin- releasing hormone receptor 1alpha (CRH-1alpha) and the CRH-C variant receptor *Journal of Clinical Endocrinology & Metabolism* (1998) **83**: 1376-1379.

Katahira, M., Iwasaki, Y., Aoki, Y., Oiso, Y., and Saito, H. Cytokine regulation of the rat proopiomelanocortin gene expression in AtT-20 cells. *Endocrinology* (1998) **139**: 2414-2422.

Katsuura, G., Arimura, A., Koves, K., and Gottschall, P. E. Involvement of organum vasculosum of lamina terminalis and preoptic area in interleukin 1beta-induced ACTH release *American Journal of Physiology - Endocrinology & Metabolism* (1990) **258**: E163-E171.

Katsuura, G., Gottschall, P. E., Dahl, R. R., and Arimura, A. Adrenocorticotropin release induced by intracerebroventricular injection of recombinant human interleukin-1 in rats: Possible involvement of prostaglandin. *Endocrinology* (1988) **122**: 1773-1779.

Kawata, M., Hashimoto, K., Takahara, J., and Sano, Y. Differences in the distributional pattern of CRF-, oxytocin-, and vasopressin-immunoreactive nerve fibers in the median eminence of the rat. *Cell & Tissue Research* (1983) **230**: 247-258.

Keller-Wood, M. E. and Dallman, M. F. Corticosteroid inhibition of ACTH secretion *Endocrine Reviews* (1984) **5**: 1-24.

Keller-Wood, M. and Wood, C. E. Does the ovine placenta secrete ACTH under normoxic or hypoxic conditions? *American Journal of Physiology - Regulatory Integrative & Comparative Physiology* (1991) **260**: R389-R395.

Kendrick, K., Leng, G., and Higuchi, T. Noradrenaline, dopamine and serotonin release in the paraventricular and supraoptic nuclei of the rat in response to intravenous cholecystokinin injections. *Journal of Neuroendocrinology* (1991) **3**: 139-144.

Khachaturian, H., Lewis, M. E., and Watson, S. J. Enkephalin systems in diencephalon and brainstem of the rat. *Journal of Comparative Neurology* (1983) **220**: 310-320.

Kim, J. K., Summer, S. N., Wood, W. M., and Schrier, R. W. Role of glucocorticoid hormones in arginine vasopressin gene regulation. *Biochemical and Biophysical Research* (2001) **289**: 1252-1256.

Kiss, A., Palkovits, M., and Aguilera, G. Neural regulation of corticotropin releasing hormone (CRH) and CRH receptor mRNA in the hypothalamic paraventricular nucleus in the rat. *Journal of Neuroendocrinology* (1996) **8**: 103-112.

Koenig, J. I., Snow, K., Clark, B. D., Toni, R., Cannon, J. G., Shaw, A. R., Dinarello, C. A., Reichlin, S., Lee, S. L., and Lechan, R. M. Intrinsic pituitary interleukin-1 beta is induced by bacterial lipopolysaccharide. *Endocrinology* (1990) **126**: 3053-3058.

Komaki, G., Arimura, A., and Koves, K. Effect of intravenous injection of IL-1beta on PGE2 levels in several brain areas as determined by microdialysis. *American Journal of Physiology - Endocrinology & Metabolism* (1992) **262**: E246-E251.

Kovacs, K. J. Functional neuroanatomy of the parvocellular vasopressinergic system: transcriptional responses to stress and glucocorticoid feedback. *Progress in Brain Research* (1998) **119**: 31-43.

Kovacs, K. J., Arias, C., and Sawchenko, P. E. Protein synthesis blockade differentially affects the stress- induced transcriptional activation of neuropeptide genes in parvocellular neurosecretory neurons. *Molecular Brain Research* (1998) **54**: 85-91.

Kovacs, K. J. and Sawchenko, P. E. Regulation of stress-induced transcriptional changes in the hypothalamic neurosecretory neurons *Journal of Molecular Neuroscience* (1996a) **7**: 125-33.

Kovacs, K. J. and Sawchenko, P. E. Sequence of stress-induced alterations in indices of synaptic and transcriptional activation in parvocellular neurosecretory neurons. *The Journal of Neuroscience* (1996b) **16**: 262-273.

Krahn, D. D., Gosnell, B. A., Levine, A. S., and Morley, J. E. Behavioral effects of corticotropin-releasing factor: Localization and characterization of central effects. *Brain Research* (1988) **443**: 63-69.

Kreig, W. J. S. The hypothalamus of the albino rat. *Journal of Comparative Neurology* (1932) **55**: 19-89.

Krieger, D. T. Regulation of circadian periodicity of plasma ACTH levels. *Annals of the New York Academy of Sciences* (1977) **297**: 561-567.

Kristensen, P., Judge, M. E., Thim, L., Ribel, U., Christjansen, K. N., Wulff, B. S., Clausen, J. T., Jensen, P. B., Madsen, O. D., Vrang, N., Larsen, P. J., and Hastrup, S. Hypothalamic CART is a new anorectic peptide regulated by leptin. *Nature* (1998) **393**: 72-76.

Kuru, M., Ueta, Y., Serino, R., Nakazato, M., Yamamoto, Y., Shibuya, I., and Yamashita, H. Centrally administered orexin/hypocretin activates HPA axis in rats. *Neuroreport* (2000) **11**: 1977-1980.

Kwak, S. P., Young, E. A., Morano, I., Watson, S. J., and Akil, H. Diurnal corticotropin-releasing hormone mRNA variation in the hypothalamus exhibits a rhythm distinct from that of plasma corticosterone. *Neuroendocrinology* (1992) **55**: 74-83.

Lacroix, S., Feinstein, D., and Rivest, S. The bacterial endotoxin lipopolysaccharide has the ability to target the brain in upregulating its membrane CD14 receptor within specific cellular populations. *Brain Pathology* (1998) **8**: 625-640.

Lacroix, S. and Rivest, S. Effect of acute systemic inflammatory response and cytokines on the transcription of the genes encoding cyclooxygenase enzymes (COX-1 and COX-2) in the rat brain. *Journal of Neurochemistry* (1998) **70**: 452-466.

Lacroix, S., Vallieres, L., and Rivest, S. C-fos mRNA pattern and corticotropin-releasing factor neuronal activity throughout the brain of rats injected centrally with a prostaglandin of E2 type. *Journal of Neuroimmunology* (1996) **70**: 163-179.

Ladd, C. O., Huot, R. L., Thrivikraman, K. V., Nemeroff, C. B., Meaney, M. J., and Plotsky, P. M. Long-term behavioral and neuroendocrine adaptations to adverse early experience. *Progress in Brain Research.* (2000) **122**: 81-103.

LaFond, R. E., Kennedy, S. W., Harrison, R. W., and Villee, C. A. Immunocytochemical localization of glucocorticoid receptors in cells, cytoplasts, and nucleoplasts. *Experimental Cell Research* (1988) **175**: 52-62.

Lakshmi, V. and Monder, C. Purification and characterization of the corticosteroid 11beta-dehydrogenase component of the rat liver 11beta-hydroxysteroid dehydrogenase complex. *Endocrinology* (1988) **123**: 2390-2398.

Lambert, J. J., Belelli, D., Hill-Venning, C., and Peters, J. A. Neurosteroids and GABA(A) receptor function. *Trends in Pharmacological Sciences* (1995) **16**: 295-303.

Lang, R. E., Heil, J. W. E, Ganten, D, Hermann, K, Unger, T, and Rascher, W Oxytocin Unlike Vasopressin Is A Stress Hormone In The Rat. *Neuroendocrinology* (1983) **37**: 314-316.

Laugero, K. D. A new perspective on glucocorticoid feedback: Relation to stress, carbohydrate feeding and feeling better. *Journal of Neuroendocrinology* (2001) **13**: 827-835.

Laugero, K. D., Bell, M. E., Bhatnagar, S., Soriano, L., and Dallman, M. F. Sucrose ingestion normalizes central expression of corticotropin-releasing-factor messenger ribonucleic acid and energy balance in adrenalectomized rats: A glucocorticoid-metabolic-brain axis? *Endocrinology* (2001) **142**: 2796-2804.

Law, S. W., Conneely, O. M., DeMayo, F. J., and O'Malley, B. W. Identification of a new brain-specific transcription factor, Nurr1. *Molecular Endocrinology* (1992) **6**: 2129-2135.

Lawrence, C. B., Turnbull, A. V., and Rothwell, R. J. Hypothalamic control of feeding. *Current Opinion in Neurobiology* (1999) **9**: 778-783.

Lee, Y. and Davis, M. Role of the hippocampus, the bed nucleus of the stria terminalis, and the amygdala in the excitatory effect of corticotropin-releasing hormone on the acoustic startle reflex. *Journal of Neuroscience* (1997) **17** : 6434-6446.

LeMay, L. G., Vander, A. J., and Kluger, M. J. The effects of psychological stress on plasma interleukin-6 activity in rats. *Physiology & Behavior* (1990) **47**: 957-961.

Lenczowski, M. J. P., Van Dam, A. M., Poole, S., Larrick, J. W., and Tilders, F. J. H. Role of circulating endotoxin and interleukin-6 in the ACTH and corticosterone response to intraperitoneal LPS. *American Journal of Physiology - Regulatory Integrative & Comparative Physiology* (1997) **273**: R1870-R1877.

Leng G. Oxytocin *Encyclopedia of Stress* (2000) **3**: 109.

Levitt, N. S., Lindsay, R. S., Holmes, M. C., and Seckl, J. R. Dexamethasone in the last week of pregnancy attenuates hippocampal glucocorticoid receptor gene expression and elevates blood pressure in the adult offspring in the rat. *Neuroendocrinology* (1996) **64**: 412-418.

Li, C., Chen, P., and Smith, M. S. Corticotropin releasing hormone neurons in the paraventricular nucleus are direct targets for neuropeptide Y neurons in the arcuate nucleus: An anterograde tracing study. *Brain Research* (2000) **854**: 122-129.

Li, C. H., Evans, H., and Simpson, M. E. Adrenocorticotropic Hormone. *Journal of Biological Chemistry* (1943) **149**: 413-414.

Li, H. Y., Ericsson, A., and Sawchenko, P. E. Distinct mechanisms underlie activation of hypothalamic neurosecretory neurons and their medullary catecholaminergic afferents in categorically different stress paradigms. *Proceedings of the National Academy of Sciences of the United States of America* (1996) **93**: 2359-2364.

Liang, K. C., Melia, K. R., Miserendino, M. J. D., Falls, W. A., Campeau, S., and Davis, M. Corticotropin-releasing factor: Long-lasting facilitation of the acoustic startle reflex. *Journal of Neuroscience* (1992) **12**: 2303-2312.

Lightman, S. L. and Harbuz, M. S. Expression of corticotropin releasing factor mRNA in response to stress. *In Corticotropin-Releasing Factor (Eds. Chadwick, D.J. et al)* (1993) 173-188.

Lightman, S. L. and Young, W. S. III Vasopressin, oxytocin, dynorphin, enkephalin and corticotrophin-releasing factor mRNA stimulation in the rat. *Journal of Physiology* (1987) **394**: 23-39.

Lightman, S. L. and Young, W. S. III Lactation inhibits stress-mediated secretion of corticosterone and oxytocin and hypothalamic accumulation of corticotropin-releasing factor and enkephalin messenger ribonucleic acids. *Endocrinology* (1989) **124**: 2358-2364.

Lin, L., Faraco, J., Li, R., Kadotani, H., Rogers, W., Lin, X., Qiu, X., De Jong, P. J., Nishino, S., and Mignot, E. The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. *Cell* (1999) **98**: 365-376.

Lindsay, R. S., Benediktsson, R., Noble, J., Seckl, J., and Edwards, C. R. W. Dexamethasone treatment of pregnant rats leads to raised blood pressure in the offspring. *Journal of Hypertension* (1992) **10**: 1431-1432.

Lindsay, R. S., Lindsay, R. M., Edwards, C. R. W., and Seckl, J. R. Inhibition of 11beta-hydroxysteroid dehydrogenase in pregnant rats and the programming of blood pressure in the offspring. *Hypertension* (1996a) **27**: 1200-1204.

Lindsay, R. S., Lindsay, R. M., Waddell, B. J., and Seckl, J. R. Prenatal glucocorticoid exposure leads to offspring hyperglycaemia in the rat: Studies with the 11 beta-hydroxysteroid dehydrogenase inhibitor carbenoxolone. *Diabetologia* (1996b) **39**: 1299-1305.

Linton, E. A. and Lowry, P. J. A large molecular weight carrier for CRF-41 in human plasma. *Journal of Endocrinology* (1986) **111** (suppl)

Linton, E. A., McLean, C., Nieuwenhuyzen Kruseman, A. C. and others Direct measurement of human plasma corticotropin-releasing hormone by 'two-site' immunoradiometric assay. *Journal of Clinical Endocrinology & Metabolism* (1987) **64** : 1047-1053.

Linton, E. A., Tilders, F. J. H., Hodgkinson, S. and others Stress-induced secretion of adrenocorticotropin in rats is inhibited by administration of antisera to ovine corticotropin-releasing factor and vasopressin. *Endocrinology* (1985) **116**: 966-970.

Liposits, Z. S., Sievers, L., and Paull, W. K. Neuropeptide-Y and ACTH-immunoreactive innervation of corticotropin releasing factor (CRF)-synthesizing neurons in the hypothalamus of the rat. An immunocytochemical analysis at the light and electron microscopic levels. *Histochemistry* (1988) **88**: 227-234.

Login. I.S and MacLeod, R. M. Prolactin in human and rat serum and cerebrospinal fluid. *Brain Research* (1977) **132**: 477-483.

Lohrenz, F. N., Seal, U. S., and Doe, R. P. Adrenal function and serum protein concentrations in a kindred with decreased corticosteroid-binding globulin (CBG) concentration. *Journal of Clinical Endocrinology and Metabolism* (1967) **27**: 966-972.

Lolait, S. J., Mezey, E., O'Carroll, A.-M., Mahan, L. C., Felder, C. C., Button, O. C., Young, W. S. III, and Brownstein, M. J. Extrapituitary expression of the rat V1b vasaopressin receptor gene. *Proceedings of the National Academy of Sciences of the USA* (1994) **92**: 6783-6787.

Lolait, S. J., O'Carroll, A. M., McBride, O. W., Konig, M., Morel, A., and Brownstein, M. J. Cloning and characterization of a vasopressin V2 receptor and possible link to nephrogenic diabetes insipidus. *Nature* (1992) **357**: 336-339.

Lovenberg, T. W., Chalmers, D. T., Liu, C., and De Souza, E. B. CRF(2alpha) and CRF(2beta) receptor mRNAs are differentially distributed between the rat central nervous system and peripheral tissues. *Endocrinology* (1995) **136**: 4139-4142.

Low, S. C., Chapman, K. E., Edwards, C. R. W., and Seckl, J. R. 'Liver-type' 11beta-hydroxysteroid dehydrogenase cDNA encodes reductase but not dehydrogenase activity in intact mammalian COS-7 cells. *Journal of Molecular Endocrinology* (1994) **13**: 167-174.

Loxley, H. D., Cowell, A. M., Flower, R. J., and Buckingham, J. C. Modulation of the hypothalamo-pituitary-adrenocortical responses to cytokines in the rat by lipocortin 1 and glucocorticoids: A role for lipocortin 1 in the feedback inhibition of CRF-41 release? *Neuroendocrinology* (1993) **57**: 801-814.

Luckman, S. M. Fos-like immunoreactivity in the brainstem of the rat following peripheral administration of cholecystokinin. *Journal of Neuroendocrinology* (1992) **4**: 149-152.

Luttge, W. G., Davda, M. M., Rupp, M. E., and Kang, C. G. High affinity binding and regulatory actions of dexamethasone-Type I receptor complexes in mouse brain. *Endocrinology* (1989) **125**: 1194-1203.

Lutz-Bucher, B., Kovacs, K., and Makara, G. Central nervous system control of pituitary vasopressin receptors: Evidence for involvement of multiple factors. *Neuroendocrinology* (1986) **43**: 618-624.

Ma, X-M, Levy, A, and Lightman, S. L. Emergence of an isolated arginine vasopressin (AVP) response to stress after repeated restraint: A study of both AVP and corticotropin-releasing hormone messenger ribonucleic acid (RNA) and heteronuclear RNA. *Endocrinology* (1997a) **138**: 4351-4357.

Ma, X-M, Levy, A, and Lightman, S. L. Rapid changes in heteronuclear RNA for corticotrophin-releasing hormone and arginine vasopressin in response to acute stress. *Journal of Endocrinology* (1997b) **152**: 81-89.

Ma, X-M and Lightman, S. L. The arginine vasopressin and corticotrophin-releasing hormone gene transcription responses to varied frequencies of repeated stress in rats. *Journal of Physiology* (1998) **510**: 605-614.

Ma, X. M., Lightman, S. L., and Aguilera, G. Vasopressin and corticotropin-releasing hormone gene responses to novel stress in rats adapted to repeated restraint. *Endocrinology* (1999) **140**: 3623-3632.

Maccari, S., Mormede, P., Piazza, P. V., Simon, H., Angelucci, L., and Le Moal, M. Hippocampal type I and type II corticosteroid receptors are modulated by central noradrenergic systems. *Psychoneuroendocrinology* (1992) **17**: 103-112.

Magiakou, M. A., Mastorakos, G., Rabin, D., Margioris, A. N., Dubbert, B., Calogero, A. E., Tsigos, C., Munson, P. J., and Chrousos, G. P. The maternal hypothalamic-pituitary-adrenal axis in the third trimester of human pregnancy. *Clinical Endocrinology* (1996) **44**: 419-428.

Majewska, M. D. Neurosteroids: Endogenous bimodal modulators of the GABA(A) receptor. Mechanism of action and physiological significance. *Progress in Neurobiology* (1992) **38**: 379-395.

Makino, S., Gold, P. W., and Schulkin, J. Corticosterone effects on corticotropin-releasing hormone mRNA in the central nucleus of the amygdala and the parvocellular region of the paraventricular nucleus of the hypothalamus. *Brain Research* (1994) **640**: 105-112.

Makino, S., Schulkin, J., Smith, M. A., Pacak, K., Palkovits, M., and Gold, P. W. Regulation of corticotropin-releasing hormone receptor messenger ribonucleic acid in the rat brain and pituitary by glucocorticoids and stress. *Endocrinology* (1995) **136**: 4517-4525.

Malkoski, S. P. and Dorin, R. I. Composite glucocorticoid regulation at a functionally defined negative glucocorticoid response element of the human corticotropin-releasing hormone gene. *Molecular Endocrinology* (1999) **13**: 1629-1644.

Mansi, J. A., Rivest, S, and Drolet, G Effect of immobilization stress on transcriptional activity of inducible immediate-early genes, corticotropin-releasing factor, its type 1 receptor, and enkephalin in the hypothalamus of borderline hypertensive rats. *Journal of Neurochemistry* (1998) **70**: 1556-1566.

Mansour, A., Fox, C. A., Burke, S., Meng, F., Thompson, R. C., Akil, H., and Watson, S. J. Mu, delta, and kappa opioid receptor mRNA expression in the rat CNS: An in situ hybridization study. *Journal of Comparative Neurology* (1994) **350**: 412-438.

Mansour, A., Khachaturian, H., Lewis, M. E., Akil, H., and Watson, S. J. Autoradiographic differentiation of mu, delta, and kappa opioid receptors in the rat forebrain and midbrain. *Journal of Neuroscience* (1987) **7**: 2445-2464.

Martinez, M., Phillips, P. J., and Herbert, J. Adaptation in patterns of c-fos expression in the brain associated with exposure to either single or repeated social stress in male rats. *European Journal of Neuroscience* (1998) **10**: 20-33.

Maruyama, K, Tsukada, T, Ohkura, N, Bandoh, S, Hosono, T, and Yamaguchi, K The NGFI-B subfamily of the nuclear receptor superfamily (Review). *International Journal of Oncology* (1998) **12**: 1237-1243.

Matsumura, K., Watanabe, Y., Imai-Matsumura, K., Connolly, M., Koyama, Y., Onoe, H., and Watanabe, Y. Mapping of prostaglandin E2 binding sites in rat brain using quantitative autoradiography. *Brain Research* (1992) **581**: 292-298.

Matsumura, K., Watanabe, Y., Onoe, H., Watanabe, Y., and Hayaishi, O. High density of prostaglandin E2 binding sites in the anterior wall of the 3rd ventricle: A possible site of its hyperthermic action. *Brain Research* (1990) **533**: 147-151.

Matta, S. G., Singh, J., Newton, R., and Sharp, B. M. The adrenocorticotropin response to interleukin-1beta instilled into the rat median eminence depends on the local release of catecholamines. *Endocrinology* (1990) **127**: 2175-2182.

McCabe, J. T. and Pfaff, D. W. In situ Hybridisation: A Methodological Guide *Methods in Neurosciences* (1989) **1**: 98-126.

McCann, S. M. and Brobeck, J. R. Evidence for a role of the supraopticohypophyseal system in the regulation of adrenocorticotropin secretion. *Proceedings of the National academy of Sciences of the USA* (1954) **87**: 318-324.

McCarty, R. Regulation of plasma catecholamine responses to stress. *Seminars in the Neurosciences* (1994) **6**: 197-204.

McCormick, C. M., Smythe, J. W., Sharma, S., and Meaney, M. J. Sex-specific effects of prenatal stress on hypothalamic-pituitary-adrenal responses to stress and brain glucocorticoid receptor density in adult rats. *Brain Research:Developmental Brain Research.* (1995) **84**: 55-61

McEwen, B. S., De Kloet, E. R., and Rostene, W. Adrenal steroid receptors and actions in the nervous system. *Physiological Reviews* (1986) **66**: 1121-1188.

McKinley, M. J., Burns, P., Colvill, L. M., Oldfield, B. J., Wade, J. D., Weisinger, R. S., and Tregear, G. W. Distribution of Fos immunoreactivity in the lamina terminalis and hypothalamus induced by centrally administered relaxin in conscious rats. *Journal of Neuroendocrinology* (1997) **9**: 431-7.

McKinley, M. J., Pennington, G. L., and Oldfield, B. J. Anteroventral wall of the third ventricle and dorsal lamina terminalis: headquarters for control of body fluid homeostasis? *Clinical and Experimental Pharmacology and Physiology* (1996) **23**: 271-81.

McLean, M. and Smith, R. Corticotropin-releasing hormone in human pregnancy and parturition *Trends in Endocrinology & Metabolism* (1999) **10**: 174-178.

Meaney, M. J., Tannenbaum, B., Francis, D., Bhatnagar, S., Shanks, N., Viau, V., O'Donnell, D., and Plotsky, P. M. Early environmental programming hypothalamic-pituitary-adrenal responses to stress. *Seminars in the Neurosciences* (1994) **6**: 247-259.

Meddle, S. L., Francis, K., Bishop, V. R., and Russell, J. A. Effects of pregnancy, parturition and lactaion on somatostatin, proenkephalin A and mu-opioid receptors. *British Neuroscience Association Abstracts* (2001) **16**: 48.06.

Meddle, S. L., Leng, G., Selvarajah, J. R., Bicknell, R. J., and Russell, J. A. Direct pathways to the supraoptic nucleus from the brainstem and the main olfactory bulb are activated at parturition in the rat. *Neuroscience* (2000) **101**: 1013-1021.

Melia, K. R., Ryabinin, A. E., Schroeder, R., Bloom, F. E., and Wilson, M. C. Induction and habituation of immediate early gene expression in rat brain by acute and repeated restraint stress. *Journal of Neuroscience* (1994) **14**: 5929-5938.

Melik Parsadaniantz, S., Gaillet, S., Malaval, F., Lenoir, V., Batsche, E., Barbanel, G., Gardier, A., Terlain, B., Jacquot, C., Szafarczyk, A., Assenmacher, I., and Kerdelhue, B. Lesions of the afferent catecholaminergic pathways inhibit the temporal activation of the CRH and POMC gene expression and ACTH release induced by human interleukin-1beta in the male rat. *Neuroendocrinology* (1995) **62**: 586-595.

Mendel, C. M. The free hormone hypothesis: A physiologically based mathematical model. *Endocrine Reviews* (1989) **10**: 232-274.

Merchenthaler, I., Vigh, S., Petrusz, P., and Schally, A. V. Immunocytochemical localization of corticotropin-releasing factor (CRF) in the rat brain. *American Journal of Anatomy* (1982) **165**: 385-396.

Meunier, J. C., Mollereau, C., Toll, L., Suaudeau, C., Moisand, C., Alvinerie, P., Butour, J. L., Guillemott, J. C., Ferrara, P., Monsarrat, B., Mazarguil, H., Vassart, G., Parmentier, M., and Costentin, J. Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. *Nature* (1995) **377**: 532-535.

Milkovic S, Milkovic K Paunovic J. The initiation of fetal adrenocorticotrophic activity in the rat. *Endocrinology* (1973) **92**: 380-384.

Millan, M. A., Jacobowitz, D. M., Hauger, R. L. and others Distribution of corticotropin-releasing factor receptors in primate brain. *Proceedings of the National Academy of Sciences of the United States of America* (1986) **83**: 1921-1925.

Minami, M., Kuraishi, Y., Yamaguchi, T., Nakai, S., Hirai, Y., and Satoh, M. Immobilization stress induces interleukin-1beta mRNA in the rat hypothalamus. *Neuroscience Letters* (1991) **123**: 254-256.

Moga, M. M., Saper, C. B., and Gray, T. S. Bed nucleus of the stria terminalis: Cytoarchitecture, immunohistochemistry, and projection to the parabrachial nucleus in the rat. *Journal of Comparative Neurology* (1989) **283**: 315-332.

Mohr, E. and Richter, D. Sequence analysis of the promoter region of the rat vasopressin gene. *FEBS Letters* (1990) **260**: 305-308.

Moisan, M. P., Seckl, J. R., Brett, L. P., Monder, C., Agarwal, A. K., White, P. C., and Edwards, C. R. W. 11Beta-hydroxysteroid dehydrogenase messenger ribonucleic acid expression, bioactivity and immunoreactivity in rat cerebellum. *Journal of Neuroendocrinology* (1990a) **2**: 853-858.

Moisan, M. P., Seckl, J. R., and Edwards, C. R. W. 11beta-Hydroxysteroid dehydrogenase bioactivity and messenger RNA expression in rat forebrain: Localization in hypothalamus, hippocampus, and cortex. *Endocrinology* (1990b) **127**: 1450-1455.

Moldow, R. L., Kastin, A. J., Graf, M., and Fischman, A. J. Stress mediated changes in hypothalamic corticotropin releasing factor-like immunoreactivity. *Life Sciences* (1987) **40**: 413-418.

Monder, C., Stewart, P. M., Lakshmi, V., Valentino, R., Burt, D., and Edwards, C. R. W. Licorice inhibits corticosteroid 11beta-dehydrogenase of rat kidney and liver: In vivo and in vitro studies. *Endocrinology* (1989) **125**: 1046-1053.

Montano, M. M., Wang, M. H., and Vom Saal, F. S. Sex differences in plasma corticosterone in mouse fetuses are mediated by differential placental transport from the mother and eliminated by maternal adrenalectomy or stress. *Journal of Reproduction & Fertility* (1993) **99**: 283-290.

Moore, R. Y. and Eichler, V. B. Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. *Brain Research* (1972) **42**: 201-206.

Morel, A., O'Carroll, A. M., Brownstein, M. J., and Lolait, S. J. Molecular cloning and expression of a rat Vla arginine vasopressin receptor. *Nature* (1992) **356**: 523-526.

Morgane, P. J. and Panksepp, J. (Eds) *The Handbook of the Hypothalamus* (1980) **3**:

Morimoto, A., Watanabe, T., Morimoto, K., Nakamori, T., and Murakami, N. Possible involvement of prostaglandins in psychological stress-induced responses in rats *Journal of Physiology* (1991) **443**: 421-429.

Morris, R. G. M., Garrud, P., Rawlins, J. N. P., and O'Keefe, J. Place navigation impaired in rats with hippocampal lesions. *Nature* (1982) **297**: 681-683.

Muglia, L., Jacobson, L., and Majzoub, J. A. Production of corticotropin-releasing hormone-deficient mice by targeted mutation in embryonic stem cells. *Annals of the New York Academy of Sciences.* (1996) **780**: 49-59.

Muglia, L. J., Bethin, K. E., Jacobson, L., Vogt, S. K., and Majzoub, J. A. Pituitary-adrenal axis regulation in CRH-deficient mice. *Endocrine Research* (2000) **26**: 1057-1066.

Muhtashan S, Mondal, M. S., Nakazato, M., and Matsukura, S. Orexins (hypocretins): Novel hypothalamic peptides with divergent functions. *Biochemical Cell Biology* (2000) **78**: 299-305.

Mulla, A. and Buckingham, J. C. Regulation of the hypothalamo-pituitary-adrenal axis by cytokines. *Bailliere's Best Practice and Research in Clinical Endocrinology and Metabolism,* (1999) **13**: 503-521.

Munck, A., Mendel, D. B., Smith, L. I., and Orti, E. Glucocorticoid receptors and actions. *American Review of Respiratory Disease* (1990) **141**: S2-S10.

Murakami, K., Akana, S., Dallman, M. F., and Ganong, W. F. Correlation between the stress-induced transient increase in corticotropin-releasing hormone content of the median eminence of the hypothalamus and adrenocorticotropic hormone secretion. *Neuroendocrinology* (1989) **49**: 233-241.

Muramami, N., Fukata, J., Tsukada, T., Kobayashi, H., Ebisui, O., Segawa, H. Muro S., Imura, H., and Nakao, K. Bacterial lipopolysaccharide-induced expression of interleukin-6 messenger ribonucleic acid in the rat hypothalamus, pituitary, adrenal gland, and spleen. *Endocrinology* (1993) **133**: 2574-2578.

Murphy, E. P., Dobson, A. D. W., Keller, C. H., and Conneely, O. M. Differential regulation of transcription by the nurr1/nur77 subfamily of nuclear transcription factors. *Gene Expression* (1995) **5**: 169-179.

Murphy, E. P. and Conneely, O. M. Neuroendocrine regulation of the hypothalamic pituitary adrenal axis by the nurr1/nur77 subfamily of nuclear receptors. *Molecular Endocrinology* (1997) **11**: 39-47.

Nappi, R. E. and Rivest, S. Stress-induced genetic expression of a selective corticotropin-releasing factor-receptor subtype within the rat ovaries: An effect dependent on the ovulatory cycle. *Biology of Reproduction* (1995) **53**: 1417-1428.

Naruse, G. Maternal behavior. Neuroendocrinological mechanisms in animals. *Encephale* (1997) **23**: 380-384.

Navarra, P., Pozzoli, G., Brunetti, L., Ragazzoni, E., Besser, M., and Grossman, A. Interleukin-1beta and interleukin-6 specifically increase the release of prostaglandin E2 from rat hypothalamic explants in vitro. *Neuroendocrinology* (1992) **56**: 61-68.

Navarra, P., Tsagarakis, S., Faria, M. S., Rees, L. H., Besser, G. M., and Grossman, A. B. Interleukins-1 and -6 stimulate the release of corticotropin-releasing hormone-41 from rat hypothalamus in vitro via the eicosanoid cyclooxygenase pathway. *Endocrinology* (1991) **128**: 37-44.

Neumann, I. D, Johnstone, H. A., Hatzinger, M, Liebsch, G, Shipston, M, Russell, J. A., Landgraf, R, and Douglas, A. J. Attenuated neuroendocrine responses to emotional and physical stressors in pregnant rats involve adenohypophysial changes. *Journal of Physiology* (1998) **508**: 289-300.

Neumann, I. D., Toschi, N., Ohl, F., Torner, L., and Kromer, S.A. Maternal defence as an emotional stressor in female rats: Correlation of neuroendocrine and behavioural parameters and involvement of brain oxytocin. *European Journal of Neuroscience* (2001) **13**: 1016-1024.

Neveu, P. J. and Liege, S. Mechanisms of behavioral and neuroendocrine effects of Interleukin-1 in mice. *Annals of the New York Academy of Sciences.* (2000) **917**: 175-185.

Nicholson, G., Greeley Jr, G. H., Humm, J. and others Prolactin in cerebrospinal fluid: A probable site of prolactin autoregulation. *Brain Research* (1980) **190**: 447-457.

Niimi, M., Sato, M., Wada, Y., Takahara, J., and Kawanishi, K. Effect of central and continuous intravenous injection of interleukin-1beta on brain c-fos expression in the rat: Involvement of prostaglandins. *Neuroimmunomodulation* (1996) **3**: 87-92.

Niimi, M., Wada, Y., Sato, M., Takahara, J., and Kawanishi, K. Effect of continuous intravenous injection of interleukin-6 and pretreatment with cyclooxygenase inhibitor on brain c-fos expression in the rat. *Neuroendocrinology* (1997) **66**: 47-53.

Nishioka, T., Anselmo-Franci, J. A., Li, P., Callahan, M. F., and Morris, M. Stress increases oxytocin release within the hypothalamic paraventricular nucleus. *Brain Research* (1998) **781**: 57-61.

Odio, M. and Brodish, A. Central but not peripheral opiate receptor blockade prolonged pituitary-adrenal responses to stress. *Pharmacology, Biochemistry & Behavior* (1990) **35**: 963-969.

Ohmichi, M., Hirota, K., Koike, K., Kurachi, H., Ohtsuka, S., Matsuzaki, N., Yamaguchi, M., Miyake, A., and Tanizawa, O. Binding sites for interleukin-6 in the anterior pituitary gland. *Neuroendocrinology* (1992) **55**: 199-203.

Oitzl, M. S. and De Kloet, E. R. Selective corticosteroid antagonists modulate specific aspects of spatial orientation learning. *Behavioral Neuroscience* (1992) **106**: 62-71.

Oitzl, M. S., Van Haarst, A. D., and De Kloet, E. R. Behavioral and neuroendocrine responses controlled by the concerted action of central mineralocorticoid (MRS) and glucocorticoid receptors (GRS). *Psychoneuroendocrinology* (1997) **22**: S87-S93.

Okabe, T., Takayanagi, R., Adachi, M., Imasaki, K., and Nawata, H. Nur77, a member of the steroid receptor superfamily, antagonizes negative feedback of ACTH synthesis and secretion by glucocorticoid in pituitary corticotrope cells. *Journal of Endocrinology* (1998) **156**: 169-175.

Olschowka, J. A., O'Donohue, T. L., Mueller, G. P., and Jacobowitz, D. M. The distribution of corticotropin releasing factor-like immunoreactive neurons in rat brain. *Peptides* (1982) **3**: 995-1015.

Olson, G. A., Olson, R. D., and Kastin, A. J. Endogenous opiates. *Peptides* (1995) **16**: 1517-1555.

Onaka, T., Luckman, S. M., Antonijevic, I., Palmer, R., and Leng, G. Involvement of the noradrenergic afferents from the nucleus tractus solitarii to the supraoptic nucleus in oxytocin release after peripheral cholecystokinin octapeptide in the rat. *Neuroscience* (1995) **66**: 403-412.

Ono, N., Bedran De Castro, J. C., and McCann, S. M. Ultrashort-loop positive feedback of corticotropin (ACTH)-releasing factor to enhance ACTH release in stress. *Proceedings of the National Academy of Sciences of the United States of America* (1985) **82**: 3528-3531.

Owens, M. J., Bartolome, J., Schanberg, S. M., and Nemeroff, C. B. Corticotropin-releasing factor concentrations exhibit an apparent diurnal rhythm in hypothalamic and extrahypothalamic brain regions: Differential sensitivity to corticosterone. *Neuroendocrinology* (1990) **52**: 626-631.

Pacak, K., Palkovits, M., Makino, S., Kopin, I. J., and Goldstein, D. S. Brainstem hemisection decreases corticotropin-releasing hormone mRNA in the paraventricular nucleus but not in the central amygdaloid nucleus. *Journal of Neuroendocrinology* (1996) **8**: 543-551.

Palchaudhuri, M. R., Wille, S., Mevenkamp, G., Spiess, J., Fuchs, E., and Dautzenberg, F. M. Corticotropin-releasing factor receptor type 1 from Tupaia belangeri cloning, functional expression and tissue distribution. *European Journal of Biochemistry* (1998) **258**: 78-84.

Palkovits, M. Neural pathways involved in ACTH regulation *Annals of the New York Academy of Sciences* (1977) **297**: 455-476.

Pardue, M. L. and Gall, J. G. Molecular hybridisation of radioactive DNA to the DNA of cytological preparations. *Proceedings of the National Academy of Science USA* (1969) **64**: 600-604.

Parkes, D, Rivest, S, Lee, S, Rivier, C, and Vale, W Corticotropin-Releasing Factor Activates c-fos, NGFI-B, And Corticotropin-Releasing Factor Gene-Expression Within The Paraventricular Nucleus Of The Rat Hypothalamus. *Molecular Endocrinology* (1993) **7**: 1357-1367.

Paskitti, M. E., McCreary, B. J., and Herman, J. P. Stress regulation of adrenocorticosteroid receptor gene transcription and mRNA expression in rat hippocampus: Time-course analysis. *Molecular Brain Research* (2000) **80**: 142-152.

Paul, S. M. and Purdy, R. H. Neuroactive steroids. *FASEB Journal* (1992) **6**: 2311-2322.

Pechnick, R., George, R., and Poland, R. E. Identification of multiple opiate receptors through neuroendocrine responses II. Antagonism of Mu, Kappa and Sigma agonists by naloxone and WIN 44,441-3. *Journal of Pharmacology & Experimental Therapeutics* (1985a) **232**: 170-177.

Pechnick, R., George, R., and Poland, R. E. Identification of multiple opiate receptors through neuroendocrine responses. I. Effects of agonists. *Journal of Pharmacology & Experimental Therapeutics* (1985b) **232**: 163-169.

Pellow, S. and File, S. E. Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze: A novel test of anxiety in the rat. *Pharmacology, Biochemistry & Behavior* (1986) **24**: 525-529.

Perrin, M. H., Donaldson, C. J., Chen, R., Lewis, K. A., and Vale, W. W. Cloning and functional expression of a rat brain corticotropin releasing factor (CRF) receptor. *Endocrinology* (1993) **133**: 3058-3061.

Peters, D. A. V. Prenatal stress: Effects on brain biogenic amine and plasma corticosterone levels. *Pharmacology, Biochemistry & Behavior* (1982) **17**: 721-725.

Peyron, C., Tighe, D. K., Van den Pol, A. N., De Lecea, L., Heller, H. C., Sutcliffe, J. G., and Kilduff, T. S. Neurons containing hypocretin (orexin) project to multiple neuronal systems. *Journal of Neuroscience* (1998) **18**: 9996-10015.

Pezzone, M. A., Lee, W. S., Hoffman, G. E., and Rabin, B. S. Induction of c-Fos immunoreactivity in the rat forebrain by conditioned and unconditioned aversive stimuli. *Brain Research* (1992) **597**: 41-50.

Pfaus, J. G. Neurobiology of sexual behavior. *Current Opinion in Neurobiology* (1999) **9**: 751-758.

Pfeiffer, A., Herz, A., Loriaux, D. L., and Pfeiffer, D. G. Central kappa- and mu-opiate receptors mediate ACTH-release in rats. *Endocrinology* (1985) **116**: 2688-2690.

Philips, A., Maira, M., Mullick, A., Chamberland, M., Lesage, S., Hugo, P., and Drouin, J. Antagonism between Nur77 and glucocorticoid receptor for control of transcription. *Molecular & Cellular Biology* (1997) **17**: 5952-5959.

Pi, X. J. and Grattan, D. R. Differential expression of the two forms of prolactin receptor mRNA within microdisssected hypothalamic nuclei of the rat. *Molecular Brain Research* (1998) **59**: 1-12.

Pitossi F, del Rey A Kabiersch A Besedovsky H. Induction of cytokine transcripts in the central nervous system and pituitary following peripheral administration of endotoxin to mice. *Journal of Neuroscience Research* (1997) **48**: 287-298.

Plotsky, P. M. Pathways to the secretion of adrenocorticotropin: A view from the portal. *Journal of Neuroendocrinology* (1991) **3**: 1-9.

Plotsky, P. M., Cunningham Jr, E. T., and Widmaier, E. P. Catecholaminergic modulation of corticotropin-releasing factor and adrenocorticotropin secretion. *Endocrine Reviews* (1989) **10**: 437-458.

Plotsky, P. M., Otto, S., and Sapolsky, R. M. Inhibition of immunoreactive corticotropin-releasing factor secretion into the hypophysial-portal circulation by delayed glucocorticoid feedback. *Endocrinology* (1986) **119**: 1126-30.

Plotsky, P. M., Otto, S., and Sutton, S. Neurotransmitter modulation of corticotropin releasing factor secretion into the hypophysial-portal circulation. *Life Sciences* (1987) **41**: 1311-1317.

Plotsky, P. M. and Sawchenko, P. E. Hypophysial-portal plasma levels, median eminence content, and immunohistochemical staining of corticotropin-releasing factor, arginine vasopressin, and oxytocin after pharmacological adrenalectomy. *Endocrinology* (1987) **120**: 1361-9.

Plotsky, P. M. and Vale, W. Hemorrhage-induced secretion of corticotropin-releasing factor-like immunoreactivity into the rat hypophysial portal circulation and its inhibition by glucocorticoids. *Endocrinology* (1984) **114**: 164-169.

Poltyrev, T., Keshet, G. I., Kay, G., and Weinstock, M. Role of experimental conditions in determining differences in exploratory behavior of prenatally stressed rats. *Developmental Psychobiology* (1996) **29**: 453-462.

Portanova, R. and Sayers, G. Corticosterone suppression of ACTH secretion: actinomycin D sensitive and insensitive components of the response. *Biochemical and Biophysical Research Communications* (1974) **56**: 928-933.

Potter, E., Behan, D. P., Fischer, W. H., Linton, E. A., Lowry, P. J., and Vale, W. W. Cloning and characterization of the cDNAs for human and rat corticotropin releasing factor-binding proteins. *Nature* (1991) **349**: 423-426.

Potter, E., Behan, D. P., Linton, E. A., Lowry, P. J., Sawchenko, P. E., and Vale, W. W. The central distribution of a corticotropin-releasing factor (CRF)-binding protein predicts multiple sites and modes of interaction with CRF. *Proceedings of the National Academy of Sciences of the United States of America* (1992) **89**: 4192-4196.

Priou, A., Oliver, C., and Grino, M. In situ hybridization of arginine vasopressin (AVP) heteronuclear ribonucleic acid reveals increased AVP gene transcription in the rat hypothalamic paraventricular nucleus in response to emotional stress. *Acta Endocrinologica* (1993) **128**: 466-472.

Rabadan-Diehl, C. and Aguilera, G. Glucocorticoids increase vasopressin V1b receptor coupling to phospholipase C. *Endocrinology* (1998) **139**: 3220-3226.

Rabadan-Diehl, C., Lolait, S. J., and Aguilera, G. Regulation of pituitary vasopressin V1b receptor mRNA during stress in the rat. *Journal of Neuroendocrinology* (1995) **7**: 903-910.

RabadanDiehl, C, Makara, G, Kiss, A, Zelena, D, and Aguilera, G Regulation of pituitary corticotropin releasing hormone (CRH) receptor mRNA and CRH binding during adrenalectomy: Role of glucocorticoids and hypothalamic factors. *Journal of Neuroendocrinology* (1997) **9**: 689-697.

Rajan, V., Edwards, C. R. W., and Seckl, J. R. 11beta-hydroxysteroid dehydrogenase in cultured hippocampal cells reactivates inert 11-dehydrocorticosterone, potentiating neurotoxicity. *Journal of Neuroscience* (1996) **16**: 65-70.

Rappay, G. and Makara, G. B. A quantitative approach to trace the corticotrophs in culture after adrenalectomy. *Histochemistry* (1981) **73**: 131-136.

Ray, D. and Melmed, S. Pituitary cytokine and growth factor expression and action. *Endocrine Reviews* (1997) **18**: 206-228.

Redmond, K., Douglas, A. J., Bicknell, R. J., and Russell, J. A. Evidence for plasticity in the B-endorphin innervation of the supaoptic nucleus during pregnancy in the rat. *Journal of Physiology* (1996) **495P**: 13P.

Rees, L. H., Burke, C. W., Chard, T., Evans, S. W., and Letchworth, A. T. Possible placental origin of ACTH in normal human pregnancy. *Nature* (1975) **254**: 620-622.

Reinscheid, R. K., Nothacker, H. P., Bourson, A., Ardati, A., Henningsen, R. A., Bunzow, J. R., Grandy, D. K., Langen, H., Monsma Jr, F. J., and Civelli, O. Orphanin FQ: A neuropeptide that activates an opioidlike G protein-coupled receptor. *Science* (1995) **270**: 792-794.

Reis, F.M., Fadalti, M., Florio, P., Petraglia, F. Putative role of placental corticotropin-releasing factor in the mechanisms of human parturition. *Journal of the Society for Gynecologic Investigation* (1999) **6**: 109-119.

Reisine, T. D. Cellular mechanisms regulating adrenocorticotropin release. *Journal of Receptor Research* (1984) **4**: 291-300.

Reul, J. M. H. M. and De Kloet, E. R. Two receptor systems for corticosterone in rat brain: Microdistribution and differential occupation. *Endocrinology* (1985) **117**: 2505-2511.

Reul, J. M. H. M. and De Kloet, E. R. Anatomical resolution of two types of corticosterone receptor sites in rat brain with in vitro autoradiography and computerized image analysis. *Journal of Steroid Biochemistry* (1986) **24**: 269-272.

Riley, S.C. and Challis, J.R.G. Corticotropin-releasing hormone production by the placenta and fetal membranes. *Placenta* (1991) **12**:105-119.

Rivest, S., Lacroix, S., Vallieres, L., Nadeau, S., Zhang, J., and Laflamme, N. How the blood talks to the brain parenchyma and the paraventricular nucleus of the hypothalamus during systemic inflammatory and infectious stimuli. *Experimental Biology & Medicine* (2000) **223**: 22-38.

Rivest, S. and Rivier, C. Influence of the paraventricular nucleus of the hypothalamus in the alteration of neuroendocrine functions induced by intermittent footshock or interleukin. *Endocrinology* (1991) **129**: 2049-2057.

Rivier, C. Effect of peripheral and central cytokines on the hypothalamic-pituitary-adrenal axis of the rat. *Annals of the New York Academy of Sciences* (1993) **697**: 97-105.

Rivier, C., Rivier, J., and Vale, W. Inhibition of adrenocorticotropic hormone secretion in the rat by immunoneutralization of corticotropin-releasing factor. *Science* (1982) **218**: 377-379.

Rivier, C. and Vale, W. Interaction of corticotropin-releasing factor and arginine vasopressin on adrenocorticotropin secretion in vivo. *Endocrinology* (1983a) **113**: 939-942.

Rivier, C. and Vale, W. Modulation of stress-induced ACTH release by corticotropin-releasing factor, catecholamines and vasopressin. *Nature* (1983b) **305**: 325-327.

Rivier, C. and Vale, W. Influence of corticotropin-releasing factor on reproductive functions in the rat. *Endocrinology* (1984) **114**: 914-921.

Rivier, C. and Vale, W. Effects of corticotropin-releasing factor, neurohypophyseal peptide, and catecholamines on pituitary function. *Federation Proceedings* (1985) **44**: 189-195.

Rivier, C., Vale, W., and Brown, M. In the rat, interleukin-1alpha and -beta stimulate adrenocorticotropin and catecholamine release. *Endocrinology* (1989) **125**: 3096-3102.

Rivier, J., Speiss, J., and Vale, W. Characterization of rat corticotropin releasing factor. *Proceedings of the National Academy of Sciences* (1983) **80**: 4851-4855.

Roberts, J. L., Lundblad, J. R., Eberwine, J. H., Fremeau, R. T., Salton, S. R. J., and Blum, M. Hormonal regulation of POMC gene expression in pituitary. *Annals of the New York Academy of Sciences* (1987) **512:** 275-285

Robertson, M. C. and Friesen, H. G. Two forms of rat placental lactogen revealed by radioimmunoassay. *Endocrinology* (1981) **108**: 2388-2390.

Robertson, MC, Gillespie, B., and Friesen, H. G. Characterisation of the two forms of rat placental lactogen (rPL): rPL-I and rPL-II. *Endocrinology* (1982) **111**: 1862-1866.

Robinson, B. G., Arbiser, J. L., Emanuel, R. L., and Majzoub, J. A. Species-specific placental corticotropin releasing hormone messenger RNA and peptide expression. *Molecular & Cellular Endocrinology* (1989) **62**: 337-341.

Roder, S. and Ciriello, J. Innervation of the amygdaloid complex by catecholaminergic cell groups of the ventrolateral medulla. *Journal of Comparative Neurology* (1993) **332**: 105-122.

Roder, S. and Ciriello, J. Collateral axonal projections to limbic structures from ventrolateral medullary A1 noradrenergic neurons. *Brain Research* (1994) **638**: 182-188.

Roh, M. S., Drazenovich, K. A., Barbose, J. J., Dinarello, C. A., and Cobb, C. F. Direct stimulation of the adrenal cortex by interleukin-1. *Surgery* (1987) **102**: 140-146.

Rosenblatt, J. S., Factor, E. M., and Mayer, A. D. Relationship between maternal aggression and maternal care in the rat. *Aggressive Behaviour* (1994) **20**: 243-255.

Roth, K. A., Weber, E., and Barchas, J. D. Immunoreactive corticotropin releasing factor (CRF) and vasopressin are colocalised in a subpopulation of the immunoreactive vasopressin cells in the paraventricular nucleus of the hypothalamus. *Life Sciences* (1983) **31**: 1857-1860.

Rupprecht, R. and Holsboer, F. Neuroactive steroids: Mechanisms of action and neuropsychopharmacological perspectives. *Trends in Neurosciences* (1999) **22**: 410-416.

Russell, J. A. and Leng, G. Sex, parturition and motherhood without oxytocin? *Journal of Endocrinology* (1998) **157**: 342-359.

Russell, J. A. and Douglas, A. J. Opioids. *Encyclopedia of Stress* (2000) **3**: 86-98.

Russell, S. M., Dhariwal, A. P., McCann, S. M., and Yates, F. E. Inhibition by dexamethasone of the in vivo pituitary response to corticotropin-releasing factor (CRF). *Endocrinology* (1969) **85**: 512-521.

Saito, M., Sugimoto, T., Tahara, A., and Kawashima, H. Molecular cloning and characterization of rat V1B vasopressin receptor: Evidence for its expression in extra-pituitary tissues. *Biochemical & Biophysical Research Communications* (1995) **212**: 751-757.

Sakai, K, Horiba, N, Sakai, Y, Tozawa, F, Demura, H, and Suda, T Regulation of corticotropin-releasing factor receptor messenger ribonucleic acid in rat anterior pituitary. *Endocrinology* (1996) **137**: 1758-1763.

Sakai, R. R., Lakshmi, V., Moner, C., Funder, J. W., Krowowski, z., and McEwen, B. S. Colocalisation of 11b-HSD and mineralocortocoid receptor in rat brain. *Society for Neuroscience Abstracts* (1990) 7773.

Sakurai, T., Amemiya, A., Ishii, M., Matsuzaki, I., Chemelli, R. M., Tanaka, H., Williams, S. C., Richardson, J. A., Kozlowski, G. P., Wilson, S., Arch, J. R. S., Buckingham, R. E., Haynes, A. C., Carr, S. A., Annan, R. S., McNulty, D. E., Liu W-, S., Terrett, J. A., Elshourbagy, N. A., Bergsma, D. J., and Yanagisawa, M. Orexins and orexin receptors: A family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* (1998) **92**: 573-585.

Samson, W. K., Gosnell, B., Chang, J. K., Resch, Z. T., and Murphy, T. C. Cardiovascular regulatory actions of the hypocretins in brain. *Brain Research* (1999) **831**: 248-253.

Sandi, C. The role and mechanisms of action of glucocorticoid involvement in memory storage *Neural Plasticity* (1998) **6**: 41-52.

Sapolsky, R., Rivier, C., Yamamoto, G., Plotsky, P., and Vale, W. Interleukin-1 stimulates the secretion of hypothalamic corticotropin- releasing factor. *Science* (1987) **238**: 522-4.

Sassone-Corsi, P., Visvader, J., Ferland, L., Mellon, P. L., and Verma, I. M. Induction of proto-oncogene fos transcription through the adenylate cyclase pathway: characterization of a cAMP-responsive element. *Genes Development* (1988) **2**: 1529-1538.

Sato, T., Sato, M., Shinsako, J., and Dallman, M. F. Corticosterone-induced changes in hypothalamic corticotropin-releasing factor (CRF) content after stress. *Endocrinology* (1975) **97**: 265-274.

Saucedo-Cardenas, O. and Conneely, O. M. Comparative distribution of nurr1 and nur77 nuclear receptors in the mouse central nervous system. *Journal of Molecular Neuroscience* (1996) **7**: 51-63.

Sawchenko, P. E. Adrenalectomy-induced enhancement of CRF and vasopressin immunoreactivity in parvocellular neurosecretory neurons: Anatomic, peptide, and steroid specificity. *Journal of Neuroscience* (1987) **7**: 1093-1106.

Sawchenko, P. E., Brown, E. R., Chan, R. K. W., Ericsson, A., Li, H. Y., Roland, B. L., and Kovacs, K. J. The paraventricular nucleus of the hypothalamus and the functional neuroanatomy of visceromotor responses to stress. *Progress in Brain Research* (1996) **107**: 201-222.

Sawchenko, P. E., Li, H. Y., and Ericsson, A. Circuits and mechanisms governing hypothalamic responses to stress: A tale of two paradigms. *Progress in Brain Research.* (2000) **122**: 61-78.

Sawchenko, P. E. and Swanson, L. W. Localization, colocalization, and plasticity of corticotropin-releasing factor immunoreactivity in rat brain. *Federation Proceedings* (1985) **44**: 221-227.

Sawchenko, P. E., Swanson, L. W., Steinbusch, H. W. M., and Verhofstad, A. A. J. The distribution and cells of origin of serotonergic inputs to the paraventricular and supraoptic nuclei of the rat. *Brain Research* (1983) **277**: 355-360.

Sawchenko, P. E., Swanson, L. W., and Vale, W. W. Co-expression of corticotropin-releasing factor and vasopressin immunoreactivity in parvocellular neurosecretory neurons of the adrenalectomized rat. *Proceedings of the National Academy of Sciences of the United States of America* (1984a) **81**: 1883-1887.

Sawchenko, P. E., Swanson, L. W., and Vale, W. W. Corticotropin-releasing factor: Co-expression within distinct subsets of oxytocin-, vasopressin-, and neurotensin-immunoreactive neurons in the hypothalamus of the male rat. *Journal of Neuroscience* (1984b) **4**: 1118-1129.

Sayers, G. and Sayers, M. A. Regulation of pituitary adrenocorticotropic activity during the response of the rat to acute stress. *Endocrinology* (1947) **40**: 265.

Schachter, B. S., Johnson, L. K., Baxter, J. D., and Roberts, J. L. Differential regulation by glucocorticoids of proopiomelanocortin mRNA levels in the anterior and intermediate lobes of the rat pituitary. *Endocrinology* (1982) **110**: 1442-1444.

Schlosser, S. F., Almeida, O. F. X., Patchev, V. K., Yassouridis, A., and Elands, J. Oxytocin-stimulated release of adrenocorticotropin from the rat pituitary is mediated by arginine vasopressin receptors of the V(1b) type. *Endocrinology* (1994) **135**: 2058-2063.

Schmidt, E. D., Binnekade, R., Janszen, A. W. J. W., and Tilders, F. J. H. Short stressor induced long-lasting increases in vasopressin stores in hypothalamic corticotropin-releasing hormone (CRH) neurons in adult rats. *Journal of Neuroendocrinology* (1996) **8**: 703-712.

Schmidt, E. D., Janszen, A. W. J. W., Wouterlood, F. G., and Tilders, F. J. H. Interleukin-1-induced long-lasting changes in hypothalamic corticotropin- releasing hormone (CRH) - Neurons and hyperresponsiveness of the hypothalamus-pituitary-adrenal axis. *Journal of Neuroscience* (1995) **15**: 7417-7426.

Schneider, M. L. Prenatal stress exposure alters postnatal behavioral expression under conditions of novelty challenge in rhesus monkey infants. *Developmental Psychobiology* (1992) **25**: 529-540.

Schneider, M. L. and Coe, C. L. Repeated social stress during pregnancy impairs neuromotor development of the primate infant. *Journal of Developmental Behaviour Pediatrics* (1993) **14**: 81-87.

Schobitz, B., Voorhuis, D. A. M., and De Kloet, E. R. Localization of interleukin 6 mRNA and interleukin 6 receptor mRNA in rat brain. *Neuroscience Letters* (1992) **136**: 189-192.

Schulte, H. M., Weisner, D., and Allolio, B. The corticotrophin releasing hormone test in late pregnancy: Lack of adrenocorticotrophin and cortisol response. *Clinical Endocrinology* (1990) **33**: 99-106.

Schultzberg, M., Andersson, C., Unden, A., Troye-Blomberg, M., Svenson, S. B., and Bartfai, T. Interleukin-1 in adrenal chromaffin cells. *Neuroscience* (1989) **30**: 805-810.

Schwartz, J., Billestrup, N., Perrin, M. and others Identification of corticotrophin-releasing factor (CRF) target cells and effects of dexamethasone on binding in anterior pituitary using a fluorescent analog of CRF. *Endocrinology* (1986) **119**: 2376-2382.

Schwartz, M. W., Baskin, D. G., Bukowski, T. R., Kuijper, J. L., Foster, D., Lasser, G., Prunkard, D. E., Porte Jr, D., Woods, S. C., Seeley, R. J., and Weigle, D. S. Specificity of leptin action on elevated blood glucose levels and hypothalamic neuropeptide Y gene expression in ob/ob mice. *Diabetes* (1996a) **45**: 531-535.

Schwartz, M. W., Peskind, E., Raskind, M., Boyko, E. J., and Porte Jr, D. Cerebrospinal fluid leptin levels: Relationship to plasma levels and to adiposity in humans. *Nature Medicine* (1996b) **2**: 589-593.

Schwartz, M. W., Seeley, R. J., Woods, S. C., Weigle, D. S., Campfield, L. A., Burn, P., and Baskin, D. G. Leptin increases hypothalamic pro-opiomelanocortin mRNA expression in the rostral arcuate nucleus. *Diabetes* (1997) **46**: 2119-2123.

Schwartz, M. W., Woods, S. C., Porte, D. Jr, Seeley, R. J., and Baskin, D. G. Central nervous system control of food intake. *Nature* (2000) **404**: 661-671.

Seasholtz AF, Thompson RC Douglass JO. Identification of a cyclic adenosine monophosphate-responsive element in the rat corticotropin-releasing hormone gene. *Molecular Endocrinology* (1988) **2**: 1311-1319.

Seckl, J. R. Glucocorticoids and small babies. *Quarterly Journal of Medicine* (1994) **87**: 259-262.

Seckl, J. R. 11 beta-hydroxysteroid dehydrogenase in the brain: A novel regulator of glucocorticoid action? *Frontiers in Neuroendocrinology* (1997) **18** : 49-99.

Seckl, J. R. and Brown, R. W. 11-Beta-hydroxysteroid dehydrogenase: On several roads to hypertension. *Journal of Hypertension* (1994) **12**: 105-112.

Seckl, J. R., Dow, R. C., Low, S. C., Edwards, C. R. W., and Fink, G. The 11beta-hydroxysteroid dehydrogenase inhibitor glycyrrhetinic acid affects corticosteroid feedback regulation of hypothalamic corticotrophin-releasing peptides in rats. *Journal of Endocrinology* (1993) **136**: 471-477.

Seeman, T. E., Singer, B. H., Rowe, J. W., Horwitz, R. I., and McEwen, B. S. Price of adaptation - Allostatic load and its health consequences: MacArthur studies of successful aging. *Archives of Internal Medicine* (1997) **157**: 2259-2268.

Selye H. Syndrome produced by diverse nocous agents. *Nature* (1936) **138**: 32.

Sherlock, D. A., Field, P. M., and Raisman, G. Retrograde transport of horseradish peroxidase in the magnocellular neurosecretory system of the rat. *Brain Research* (1975) **88**: 403-414.

Shibasaki, T., Odagiri, E., Shizume, K., and Ling, N. Corticotropin-releasing factor-like activity in human placental extracts. *Journal of Clinical Endocrinology & Metabolism* (1982) **55**: 384-386.

Shintani, F., Nakaki, T., Kanba, S., Sato, K., Yagi, G., Shiozawa, M., Aiso, S., Kato, R., and Asai, M. Involvement of interleukin-1 in immobilization stress-induced increase in plasma adrenocorticotropic hormone and in release of hypothalamic monoamines in the rat. *Journal of Neuroscience* (1995) **15**: 1961-1970.

Shirley, B. The food intake of rats during pregnancy and lactation. *Laboratory Animal Science* (1984) **34**: 169-172.

Siegel, R. A., Chowers, I., Conforti, N. and others Effects of naloxone on basal and stress-induced ACTH and corticosterone secretion in the male rat. Site and mechanism of action. *Brain Research* (1982) **249**: 103-109.

Sirinathsinghji, D. J. S. Regulation of lordosis behaviour in the female rat by corticotropin-releasing factor, beta-endorphin/corticotropin and luteinizing hormone neuronal systems in the medial preoptic area. *Brain Research* (1986) **375**: 49-56.

Sirinathsinghji, D. J. S., Rees, L. H., Rivier, J., and Vale, W. Corticotropin-releasing factor is a potent inhibitor of sexual receptivity in the female rat. *Nature* (1983) **305**: 232-235.

Smelik, P. G. Relation between blood levels of corticoids an their inhibiting effect on the hypophyseal stress response. *Proceedings of the Society for Experimental and Biology and Medicine* (1963) **113**: 616.

Smith, G. P., Jerome, C., Cushin, B. J., Eterno, R., and Simansky, K. J. Abdominal vagotomy blocks the satiety effect of cholecystokinin in the rat. *Science* (1981) **213**: 1036-1037.

Smith, G. W., Aubry, J. M., Dellu, F., Contarino, A., Bilezikjian, L. M., Gold, L. H., Chen, R., Marchuk, Y., Hauser, C., Bentley, C. A., Sawchenko, P. E., Koob, G. F., Vale, W., and Lee, K. F. Corticotropin releasing factor receptor 1-deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development. *Neuron* (1998) **20**: 1093-1102.

Spencer, R. L., Miller, A. H., Moday, H., McEwen, B. S., Blanchard, R. J., Blanchard, D. C., and Sakai, R. R. Chronic social stress produces reductions in available splenic type II corticosteroid receptor binding and plasma corticosteroid binding globulin levels. *Psychoneuroendocrinology* (1996) **21**: 95-109.

Spiess, J., Rivier, J., Rivier, C., and Vale, W. Primary structure of corticotropin-releasing factor from ovine hypothalamus. *Proceedings of the National Academy of Sciences of the United States of America* (1981) **78**: 6517-6521.

Srisawat, R., Ludwig, M., Bull, P. M., Douglas, A. J., Russell, J. A., and Leng, G. Nitric oxide and the oxytocin system in pregnancy. *Journal of Neuroscience* (2000) **20**: 6721-6727.

Stanley, B. G. and Leibowitz, S. F. Neuropeptide Y: Stimulation of feeding and drinking by injection into the paraventricular nucleus. *Life Sciences* (1984) **35**: 2635-2642.

Stephan, F. K., Berkley, K. J., and Moss, R. L. Efferent connections of the rat suprachiasmatic nucleus. *Neuroscience* (1981) **6**: 2625-2641.

Stephens, T. W., Basinski, M., Bristow, P. K., Bue-Valleskey, J. M., Burgett, S. G., Craft, L., Hale, J., Hoffmann, L., Hsiung, H. M., Kriauciunas, A., MacKellar, W., Rosteck Jr, P. R., Schoner, B., Smith, D., Tinsley, F. C., Zhang, X. Y., and Heiman, M. The role of neuropeptide Y in the antiobesity action of the obese gene product. *Nature* (1995) **377**: 530-532.

Sternberg, E. M., Hill, J. M., Chrousos, G. P., Kamilaris, T., Listwak, S. J., Gold, P. W., and Wilder, R. L. Inflammatory mediator-induced hypothalamic-pituitary-adrenal axis activation is defective in streptococcal cell wall arthritis-susceptible Lewis rats. *Proceedings of the National Academy of Sciences of the United States of America* (1989a) **86**: 2374-2378.

Sternberg, E. M., Young, W. S. III, Bernardini, R., Calogero, A. E., Chrousos, G. P., Gold, P. W., and Wilder, R. L. A central nervous system defect in biosynthesis of corticotropin-releasing hormone is associated with susceptibility to streptococcal cell wall-induced arthritis in Lewis rats. *Proceedings of the National Academy of Sciences of the United States of America* (1989b) **86**: 4771-4775.

Stott, D. H. Follow-up study from birth of the effects of prenatal stresses. *Developmental Medicine and Child Neurology* (1973) **15**: 770-787.

Suda, T., Tomori, N., Yajima, F. and others Immunoreactive corticotropin-releasing factor in human plasma. *Journal of Clinical Investigation* (1985) **76** : 2026-2029.

Suda, T., Tozawa, F., Ushiyama, T., Sumitomo, T., Yamada, M., and Demura, H. Interleukin-1 stimulates corticotropin-releasing factor gene expression in rat hypothalamus. *Endocrinology* (1990) **126**: 1223-1228.

Suda, T., Tozawa, F., Iwai, I., Sato, Y., Sumitomo, T., Nakano, Y., Yamada, M., and Demura, H. Neuropeptide Y increases the corticotropin-releasing factor messenger ribonucleic acid level in the rat hypothalamus *Molecular Brain Research* (1993) **18**: 311-315.

Sugiyama, T., Minoura, H., Kawabe, N., Tanaka, M., and Nakashima, K. Preferential expression of long form prolactin receptor mRNA in the rat brain during the oestrous cycle, pregnancy and lactation: Hormones involved in its gene expression. *Journal of Endocrinology* (1994) **141**: 325-333.

Sumimoto, T., Saito, M., Mochizuki, S., Wanatabe, Y., Hashimoto, S., and Kawashima, H. Molecular cloning and functional expression of a CDNA encoding the human V1b vasopressin receptor. *Journal of Biological Chemistry* (1994) **269**: 27088-27092.

Sun, N., Roberts, L., and Cassell, M. D. Rat central amygdaloid nucleus projections to the bed nucleus of the stria terminalis. *Brain Research Bulletin* (1991) **27**: 651-662.

Sutton, R. E., Koob, G. F., Le Moal, M. and others. Corticotropin releasing factor produces behavioural activation in rats. *Nature* (1982) **297**: 331-333.

Swanson, L. W. and Kuypers, H.G.J.M. The paraventricular nucleus of the hypothalamus: Cytoarchitectonic subdivisions and organization of projections to the pituitary, dorsal vagal complex, and spinal cord as demonstrated by retrograde fluorescence double-labeling methods. *Journal of Comparative Neurology* (1980) **194**: 555-570.

Swanson, L. W. and Sawchenko, P. E. Paraventricular nucleus: A site for the integration of neuroendocrine and autonomic mechanisms. *Neuroendocrinology* (1980) **31**: 410-417.

Swanson, L. W. and Sawchenko, P. E. Hypothalamic integration: Organization of the paraventricular and supraoptic nuclei. *Annual Review of Neuroscience.* (1983) **6**: 269-324.

Swanson, L. W., Sawchenko, P. E., Lind, R. W., and Rho, J. H. The CRH motoneuron: Differential peptide regulation in neurons with possible synaptic, paracrine, and endocrine outputs. *Annals of the New York Academy of Sciences.* (1987) **512**: 12-23.

Swanson, L. W., Sawchenko, P. E., Rivier, J., and Vale, W. W. Organization of ovine corticotropin-releasing factor immunoreactive cells and fibers in the rat brain: An immunohistochemical study. *Neuroendocrinology* (1983) **36**: 165-186.

Swanson, L. W., Sawchenko, P. E., Wiegand, S. J., and Price, J. L. Separate neurons in the paraventricular nucleus project to the median eminence and to the medulla or spinal cord. *Brain Research* (1980) **198**: 190-195.

Szafarczyk, A., Alonso, G., Ixart, G. and others Serotoninergic system and circadian rhythms of ACTH and corticosterone in rats. *American Journal of Physiology - Endocrinology & Metabolism* (1980) **2**: E482-E489.

Szafarczyk, A., Alonso, G., Ixart, G. and others Diurnal-stimulated and stress-induced ACTH release in rats mediated by ventral noradrenergic bundle. *American Journal of Physiology - Endocrinology & Metabolism* (1985) **12**: E219-E226.

Szuran, T., Zimmermann, E., and Welzl, H. Water maze performance and hippocampal weight of prenatally stressed rats. *Behavioural Brain Research* (1994) **65**: 153-155.

Takahashi, L. K., Baker, E. W., and Kalin, N. H. Ontogeny of behavioral and hormonal responses to stress in prenatally stressed male rat pups. *Physiology & Behavior* (1990) **47**: 357-364.

Takahashi, L. K., Haglin, C., and Kalin, N. H. Prenatal stress potentiates stress-induced behavior and reduces the propensity to play in juvenile rats. *Physiology & Behavior* (1992) **51**: 319-323.

Takahashi, L. K. and Kalin, N. H. Early developmental and temporal characteristics of stress-induced secretion of pituitary-adrenal hormones in prenatally stressed rat pups. *Brain Research* (1991) **558**: 75-78.

Takahashi, L. K., Turner, J. G., and Kalin, N. H. Prenatal stress alters brain catecholaminergic activity and potentiates stress-induced behavior in adult rats. *Brain Research* (1992) **574**: 131-137.

Takahashi, N., Okumura, T., Yamada, H., and Kohgo, Y. Stimulation of gastric acid secretion by centrally administered orexin-A in conscious rats. *Biochemical & Biophysical Research Communications* (1999) **254**: 623-627.

Takebe, K., Kunita, H., Sakakura, M., Horiuchi, Y., and Mashimo, K. Suppressive effect of dexamethasone on the rise of CRF activity in the median eminence induced by stress. *Endocrinology* (1971) **89**: 1014-1019.

Takemura, T., Makino, S., Takao, T., Asaba, K., Suemaru, S., and Hashimoto, K. Hypothalamic-pituitary-adrenocortical responses to single vs. repeated endotoxin lipopolysaccharide administration in the rat. *Brain Research* (1997) **767**: 181-191.

Tempel, D. L., Leibowitz, K. J., and Leibowitz, S. F. Effects of PVN galanin on macronutrient selection. *Peptides* (1988) **9**: 309-314.

Thannickal, T. C., Moore, R. Y., Nienhuis, R., Ramanathan, L., Gulyani, S., Aldrich, M., Cornford, M., and Siegel, J. M. Reduced number of hypocretin neurons in human narcolepsy. *Neuron* (2000) **27**: 469-474.

Thornton, J. E., Cheung, C. C., Clifton, D. K., and Steiner, R. A. Regulation of hypothalamic proopiomelanocortin mRNA by leptin in ob/ob mice. *Endocrinology* (1997) **138**: 5063-5066.

Tilders, F. J. H., Berkenbosch, F., Vermes, I., and al Role of the epinephrine and vasopressin in the control of the pituitary-adrenal response to stress. *Federation Proceedings* (1985) **44**: 155-160.

Tomaszewska, D. and Przekop, F. The immune-neuro-endocrine interactions. *Journal of Physiology & Pharmacology* (1997) **48**: 139-158.

Tominaga, T., Fukata, J., Naito, Y., Usui, T., Murakami, N., Fukushima, M., Nakai, Y., Hirai, Y., and Imura, H. Prostaglandin-dependent in vitro stimulation of adrenocortical steroidogenesis by interleukin.s *Endocrinology* (1991) **128**: 526-531.

Torner, L., Toschi, N., Pohlinger, A., Landgraf, R., and Neumann, I. D. Anxiolytic and anti-stress effects of brain prolactin: Improved efficacy of antisense targeting of the prolactin receptor by molecular modeling. *Journal of Neuroscience* (2001) **21**: 3207-3214.

Trapp, T., Rupprecht, R., Castren, M., Reul, J.M.H.M. and Holsboer, F. Heterodimerization between mineralocorticoid and glucocorticoid receptor: A new principle of glucocorticoid action in the CNS. *Neuron* (1994) **13**: 1457-1462.

Trivedi, P., Yu, H., MacNeil, D. J., Van der Ploeg, L. H. T., and Guan, X. M. Distribution of orexin receptor mRNA in the rat brain. *FEBS Letters* (1998) **438**: 71-75.

Truss, M. and Beato, M. Steroid hormone receptors: Interaction with deoxyribonucleic acid and transcription factors. *Endocrine Reviews* (1993) **14**: 459-479.

Tsagarakis, S., Gillies, G., Rees, L. H., Besser, M., and Grossman, A. Interleukin-1 directly stimulates the release of corticotrophin releasing factor from rat hypothalamus *Neuroendocrinology* (1989a) **49**: 98-101.

Tsagarakis, S., Rees, L. H., Besser, G. M., and Grossman, A. Neuropeptide-Y stimulates CRF-41 release from rat hypothalami in vitro. *Brain Research* (1989b) **502**: 167-170.

Turnbull, A. V., Lee, S., and Rivier, C. Mechanisms of hypothalamic-pituitary-adrenal axis stimulation by immune signals in the adult rat. *Annals of the New York Academy of Sciences* (1998) **840**: 434-443.

Turnbull, A. V. and Rivier, C. Regulation of the HPA axis by cytokines. *Brain, Behavior, & Immunity* (1995) **9**: 253-275.

Uhl, G. R., Zingg, H. H., and Habener, J. F. Vasopressin mRNA in situ hybridization: localization and regulation studied with oligonucleotide cDNA probes in normal and Brattleboro rat hypothalamus. *Proceedings of the National Academy of Science U S A* (1985) **82**: 5555-5559.

Vale, W. and Rivier, C. Regulation of ACTH secretion by anterior pituitary cells in culture. *Federation Proceedings* (1976) **35**:

Vale, W. and Rivier, C. Substances modulating the secretion of ACTH by cultured anterior pituitary cells. *Federation Proceedings* (1977) **36**: 2094-2099.

Vale, W., Rivier, C., and Brown, M. Regulatory peptides of the hypothalamus. *Annual Reviews in Physiology* (1977) **39**: 473-527.

Vale, W., Rivier, C., Brown, M. R., Spiess, J., Koob, G., Swanson, L., Bilezikjian, L., Bloom, F., and Rivier, J. Chemical and Biological Characterisation of Corticotropin Releasing Factor. *Recent Progress in Hormone Research* (1983) **39**: 245-270.

Vale, W., Spiess, J., Rivier, C., and Rivier, J. Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. *Science* (1981) **213**: 1394-1397.

Valentino, R. J. and Foote, S. L. Corticotropin-releasing factor disrupts sensory responses of brain noradrenergic neurons. *Neuroendocrinology* (1987) **45**: 28-36.

Vallee, M., Mayo, W., Dellu, F., Le Moal, M., Simon, H., and Maccari, S. Prenatal stress induces high anxiety and postnatal handling induces low anxiety in adult offspring: Correlation with stress-induced corticosterone secretion. *Journal of Neuroscience* (1997) **17**: 2626-2636.

Van De Kar, L. D., Piechowski, R. A., Rittenhouse, P. A., and Gray, T. S. Amygdaloid lesions: Differential effect on conditioned stress and immobilization-induced increases in corticosterone and renin secretion. *Neuroendocrinology* (1991) **54**: 89-95.

Venihaki, M. and Majzoub, J. A. Animal models of CRH deficiency. *Frontiers in Neuroendocrinology* (1999) **20**: 122-145.

Ventura, M. A., Rene, P., De Keyzer, Y., Bertagna, X., and Clauser, E. Gene and cDNA cloning and characterization of the mouse V3/V1b pituitary vasopressin receptor. *Journal of Molecular Endocrinology* (1999) **22**: 251-260.

Verbalis, J. G., Stricker, E. M., Robinson, A. G., and Hoffman, G. E. Cholecystokinin activates c-fos expression in hypothalamic oxytocin and corticotropin-releasing hormone neurons. *Journal of Neuroendocrinology* (1991) **3**: 204-213.

Vermes, I., Mulder, G. H., and Smelik, P. G. A superfusion system technique for the study of the sites of action of glucocorticoids in the rat hypothalamus-pituitary-adrenal system in vitro. II. Hypothalamus-pituitary cell-adrenal cell superfusion. *Endocrinology 1977* (1977) **100**: 1153-1159.

Vernikos-Danellis, J. Effect of stress, adrenalectomy, hypophysectomy and hydrocortisone on the corticotroppin-releasing activity of rat median eminence. *Endocrinology* (1965) **76**: 122.

Vita, N., Laurent, P., Lefort, S., Chalon, P., Lelias, J. M., Kaghad, M., Le Fur, G., Caput, D., and Ferrara, P. Primary structure and functional expression of mouse pituitary and human brain corticotrophin releasing factor receptors. *FEBS Letters* (1993) **335**: 1-5.

Voogt, J. and De Greef, W. J. Inhibition of nocturnal prolactin surges in the pregnant rat by incubation medium containing placental lactogen. *Proceedings of the Society for Experimental Biology & Medicine* (1989) **191**: 403-407.

Waddell, B. J. The plasma as hypothalamus and pituitary: possible impact on maternal and fetal adrenal function. *Reproduction, Fertility and Development* (1993) **5**: 479-497.

Waddell, B. J. and Atkinson, H. C. Production rate, metabolic clearance rate and uterine extraction of corticosterone during rat pregnancy. *Journal of Endocrinology* (1994) **143**: 183-190.

Waddell, B. J., Benediktsson, R., Brown, R. W., and Seckl, J. R. Tissue-specific messenger ribonucleic acid expression of 11beta- hydroxysteroid dehydrogenase types 1 and 2 and the glucocorticoid receptor within rat placenta suggests exquisite local control of glucocorticoid action. *Endocrinology* (1998) **139**: 1517-1523.

Wager-Smith, K. and Kay, S.A. Circadian rhythm genetics: from flies to mice to humans. *Nature Genetics* (2000) **26**: 23-27

Wahlestedt, C., Skagerberg, G., Ekman, R., Heilig, M., Sundler, F., and Hakanson, R. Neuropeptide Y (NPY) in the area of the hypothalamic paraventricular nucleus activates the pituitary-adrenocortical axis in the rat. *Brain Research* (1987) **417**: 33-38.

Wakshlak, A. and Weinstock, M. Neonatal handling reverses behavioral abnormalities induced in rats by prenatal stress. *Physiology & Behavior* (1990) **48** : 289-292.

Ward, I. L. and Weisz, J. Differential effects of maternal stress on circulating levels of corticosterone, progesterone, and testosterone in male and female rat fetuses and their mothers. *Endocrinology* (1984) **114**: 1635-1644.

Watanabe, T., Morimoto, A., Sakata, Y., and Murakami, N. ACTH response induced by interleukin-1 is mediated by CRF secretion stimulated by hypothalamic PGE *Experientia* (1990) **46**: 481-484.

Watanobe, H., Sasaki, S., and Takebe, K. Role of prostaglandins E1, E2 and F2alpha in the brain in interleukin 1beta-induced adrenocorticotropin secretion in the rat. *Cytokine* (1995) **7**: 710-712.

Watanobe, H. and Takebe, K. Intrahypothalamic perfusion with interleukin-1-beta stimulates the local release of corticotropin-releasing hormone and arginine vasopressin and the plasma adrenocorticotropin in freely moving rats: A comparative perfusion of the paraventricular nucleus and the median eminence. *Neuroendocrinology* (1993) **57**: 593-599.

Watts, A. G. Ether anesthesia differentially affects the content of prepro-corticotropin-releasing hormone, prepro-neurotensin/neuromedin N and prepro-enkephalin mRNAs in the hypothalamic paraventricular nucleus of the rat. *Brain Research* (1991) **544**: 353-357.

Way, S. A., Douglas, A. J., Dye, S., Bicknell, R. J., Leng, G., and Russell, J. A. Endogenous opioid regulation of oxytocin release during parturition is reduced in ovariectomized rats. *Journal of Endocrinology* (1993) **138**: 13-22.

Weidenfeld, J., Abramsky, O., and Avadia, H. Evidence for the involvement of the central adrenergic system in interleukin 1-induced adrenocortical response. *Neuropharmacology* (1989) **28**: 1411-1414.

Weidenfeld, J. and Feldman, S. Effects of adrenalectomy and corticosterone replacement on the hypothalamic-pituitary response to neural stimuli. *Brain Research* (2000) **877**: 73-78.

Weinstock, M., Matlina, E., Maor, G. I., Rosen, H., and McEwen, B. S. Prenatal stress selectivity alters the reactivity of the hypothalamic-pituitary adrenal system in the female rat. *Brain Research* (1992) **595**: 195-200.

Weinstock, M., Poltyrev, T., Keshet, G. I., and Malool, R. Effect of naloxone on behavior and activation of hypothalamo-pituitary-adrenal axis in prenatally-stressed rats exposed to novel environments. *In: Stress: Molecular Genetic and Neurobiological Advances (Eds. McCart, R.; Aguilera, G.; Sabban, E.; Kvetnansky, R.* (1996)

Welberg, L. A. M. and Seckl, J. R. Prenatal stress, glucocorticoids and the programming of the brain. *Journal of Neuroendocrinology* (2001) **13**: 113-128.

Welberg, L. A. M., Seckl, J. R., and Holmes, M. C. Inhibition of 11beta-hydroxysteroid dehydrogenase, the foetoplacental barrier to maternal glucocorticoids, permanently programs amygdala GR mRNA expression and anxiety-like behaviour in the offspring. *European Journal of Neuroscience* (2000) **12**: 1047-1054.

Welberg, L. A. M., Seckl, J. R., and Holmes, M. C. Prenatal glucocorticoid programming of brain corticosteroid receptors and corticotrophin-releasing hormone: Possible implications for behaviour. *Neuroscience* (2001) **104**: 71-79.

Weller, K. L. and Smith, D. A. Afferent connections to the bed nucleus of the stria terminalis. *Brain Research* (1982) **232**: 255-270.

Whitcomb, R. W., Linehan, W. M., Wahl, L. M., and Knazek, R. A. Monocytes stimulate cortisol production by cultured human adrenocortical cells. *Journal of Clinical Endocrinology & Metabolism* (1988) **66**: 33-38.

Whitnall, M. H. Stress selectively activates the vasopressin-containing subset of corticotropin-releasing hormone neurons. *Neuroendocrinology* (1989) **50**: 702-707.

Whitnall, M. H., Mezey, E., and Gainer, H. Co-localization of corticotropin-releasing factor and vasopressin in median eminence neurosecretory vesicles. *Nature* (1985) **317**: 248-250.

Widmaier, E. P. and Dallman, M. F. Fast inhibition of stimulated ACTH secretion by corticosterone does not require protein synthesis. *Endocrinology* (1983a) **112A**: 90.

Widmaier, E. P. and Dallman, M. F. Rapid inhibition and stimulation of ACTH secretion by glucocorticoids in vitro. *Federation Proceedings* (1983b) **42**: 458.

Widmaier, E. P. and Dallman, M. F. The effects of corticotropin-releasing factor on adrenocorticotropin secretion from perifused pituitaries in vitro: Rapid inhibition by glucocorticoids. *Endocrinology* (1984) **115**: 2368-2374.

Wiegand, S. J. and Price, J. L. Cells of origin of the afferent fibers to the median eminence in the rat. *Journal of Comparative Neurology* (1980a) **192** : 1-19.

Wilson, T. E., Fahrner, T. J., Johnston, M, and Milbrandt, J Identification of the binding site for NGFI-B by genetic selection in yeast. *Science* (1991) **252**: 1296-1299.

Wilson, T. E., Fahrner, T. J., and Milbrandt, J. The orphan receptors NGFI-B and steroidogenic factor 1 establish monomer binding as a third paradigm of nuclear receptor-DNA interaction. *Molecular & Cellular Biology* (1993a) **13**: 5794-5804.

Wilson, T. E., Mouw, A. R., Weaver, C. A., Milbrandt, J., and Parker, K. L. The orphan nuclear receptor NGFI-B regulates expression of the gene encoding steroid 21-hydroxylase. *Molecular & Cellular Biology* (1993b) **13**: 861-868.

Windle, R. J., Wood, S, Shanks, N, Perks, P, Conde, G. L., da Costa, A. P. C., Ingram, C. D., and Lightman, S. L. Endocrine and behavioural responses to noise stress: Comparison of virgin and lactating female rats during non-disrupted maternal activity. *Journal of Neuroendocrinology* (1997) **9**: 407-414.

Wisden, W., Errington, M. L., Williams, S., Dunnett, S. B., Waters, C., Hitchcock, D., Evan, G., Bliss, T. V. P., and Hunt, S. P. Differential expression of immediate early genes in the hippocampus and spinal cord. *Neuron* (1990) **4**: 603-614.

Wolfson, B., Manning, R. W., Davis, L. G., Arentzen, R., and Baldino, F. Jr Co-localization of corticotropin releasing factor and vasopressin mRNA in neurones after adrenalectomy. *Nature* (1985) **315**: 59-61.

Wolvers, D. A. W., Marquette, C., Berkenbosch, F., and Haour, F. Tumor necrosis factor-alpha: Specific binding sites in rodent brain and pituitary gland. *European Cytokine Network* (1993) **4**: 377-381.

Wotjak, C. T., Kubota, M., Liebsch, G., Montkowski, A., Holsboer, F., Neumann, I., and Landgraf, R. Release of vasopressin within the rat paraventricular nucleus in response to emotional stress: A novel mechanism of regulating adrenocorticotropic hormone secretion? *Journal of Neuroscience* (1996) **16**: 7725-7732.

Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J., and Mathison, J. C. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* (1990) **249**: 1431-1433.

Wynn, P. C., Aguilera, G., Morell, J., and Catt, K. J. Properties and regulation of high-affinity pituitary receptors for corticotropin-releasing factor. *Biochemical & Biophysical Research Communications* (1983) **110**: 602-608.

Wynn, P. C., Harwood, J. P., Catt, K. J., and Aguilera, G. Regulation of corticotropin-releasing factor (CRF) receptors in the rat pituitary gland: Effects of adrenalectomy on CRF receptors and corticotroph responses. *Endocrinology* (1985) **116**: 1653-1659.

Wynn, P. C., Hauger, R. L., Holmes, M. C. and others. Brain and pituitary receptors for corticotropin releasing factor: Localization and differential regulation after adrenalectomy. *Peptides* (1984) **5**: 1077-1084.

Xu, Y., Day, T. A., and Buller, K. M. The central amygdala modulates hypothalamic-pituitary-adrenal axis responses to systemic interleukin-1beta administration. *Neuroscience* (1999) **94**: 175-183.

Yabuuchi, K., Minami, M., Katsumata, S., and Satoh, M. Localization of type I interleukin-1 receptor mRNA in the rat brain. *Molecular Brain Research* (1994) **27**: 27-36.

Yajima, F., Suda, T., Tomori, N. and others Effects of opioid peptides on immunoreactive corticotropin-releasing factor release from the rat hypothalamus in vitro. *Life Sciences* (1986) **39**: 181-186.

Yasuda, N., Takebe, K., and Greer, M. A. Studies on the corticotropin-releasing activity of vasopressin, using ACTH secretion by cultured rat adenohypophysial cells. *Endocrinology* (1976) **98**: 936-942.

Young, E. A. and Akil, H. Corticotropin-releasing factor stimulation of adrenocorticotropin and beta-endorphin release: Effects of acute and chronic stress. *Endocrinology* (1985) **117**: 23-30.

Young, W. S., Mezey, E., and Siegel, R. E. Quantitative in situ hybridization histochemistry reveals increased levels of corticotropin-releasing factor mRNA after adrenalectomy in rats. *Neuroscience Letters* (1986) **70**: 198-203.

Zardetto-Smith, A. M. and Gray, T. S. Catecholamine and NPY efferents from the ventrolateral medulla to the amygdala in the rat. *Brain Research Bulletin* (1995) **38**: 253-260.

Zhang, J. and Rivest, S. Distribution, regulation and colocalization of the genes encoding the EP2- and EP4-PGE2 receptors in the rat brain and neuronal responses to systemic inflammation. *European Journal of Neuroscience* (1999) **11**: 2651-2668.

Zhou, D., Kusnecov, A. W., Shurin, M. R., DePaoli, M., and Rabin, B. S. Exposure to physical and psychological stressors elevates plasma interleukin 6: Relationship to the activation of hypothalamic-pituitary- adrenal axis. *Endocrinology* (1993) **133**: 2523-2530.

Appendices

Suppliers

Altec

Unit 4 Rivewey Industrial Park, Alton, Hampshire, GU34 2QG, UK

Amersham

Little Chalfont, Bicks, HP7 9NA, UK.

Bantin & Kingman

Hull, HU11 4QE, UK.

Bayer Diagnostics

Hampshire, UK.

BDH

Merck House, Poole, Dorset BH15 1TD, UK.

Boeringer Mannheim Ltd.

Bell Lane, Lewes, East Sussex, BN7 1LG, UK

Bright Instrument Co. Ltd.

St. Margarets Way, Stukeley Meadows, Huntingdon, Cambs, PE 18 6EB, England

ICN Pharmaceuticals Inc.

Costa Mesa, USA

IDS Ltd.

10 Dicot Way, Boldon Business Park, Boldon, Tyne & Wear, NE35 9PD, U.K.

MWG-Biotech UK Ltd.

Waterside House, Peartree Bridge, Milton Keynes, MK6 3BY

National Institute for Biological Standards and Control

Blanche Lane, South Mimms, Potters Bar, Herts, EN6 3QC, UK.

NEN Life Science Products

BRU/BRU/40349, PO Box 66, Hounslow TW5 9RT, United Kingdom.

Novabiochem Ltd.

3 Heathcoat Building, Highfields Science Park, University Boulevard, Nottingham, NG7 2QJ, UK

Oncogene Research Products

Calbiochem (CN Biosciences), Boulevard Industrial Patk, Padge Road, Beeston, Nottingham, NG9 2JR

Packard Bioscience

Rigaweg 22, 9723TH, Groningen, Netherlands

Plastics One

Bilaney Consultants Ltd., St. Julians, Sevenoaks, Kent TN15 0RX, UK.

Qiagen

Westburg b.v., P.O. Box 214, 3830 AE Leusden, Netherlands

Sapu

Law Hospital, Lanarkshire, ML8 5ES, Scotland

Sigma

Fancy road, Poole, Dorset, BH12 4QH, UK

Vector Laboratories

3 Accent Park, Bakewell Road, Orton Southgate, Peterborough, PE2 6XS, UK.

Wallac Oy

P.O.Box 10, 20101 Turku 10, Finland

Publications

A.J. Douglas, H. Johnstone, **P. Brunton** & J.A. Russell (2000) Sex Steroid Induction of Endogenous Opioid Inhibition on Oxytocin Secretory Responses to Stress. *Journal of Neuroendocrinology* **12**; 343-350

P.J.Brunton, S. Ma, M.J. Shipston, A.Wigger, I.D.Neumann, A.J.Douglas & J.A.Russell (2000) Central Mechanisms Underlying Reduced ACTH Stress Responses in Pregnant Rats: Attenuated Acute Gene Activation in the Parvocellular Paraventricular Nucleus (pPVN). *European Journal of Neuroscience* **12**; 184.17

P.J. Brunton, S. Anderson, J. Bell & J.A. Russell (2001) Hypothalamo-Pituitary-Adrenal axis stress responses in pregnancy and glucocorticoid negative feedback. *British Neuroscience Association* **16**; P30.13

A.J. Douglas, **P.J. Brunton**, J.A. Russell & I.D. Neumann (2001) Greater attenuation of hypothalamo-pituitary-adrenal axis stress responses in pregnant vs lactating mice. *British Neuroscience Association* **16**; P30.02

P.J. Brunton, A.J. Douglas & J.A. Russell (2001) Endogenous Opioids and Attenuated Neuroendocrine Responses to Immune Challenge in Pregnant Rats. *Society for Neuroscience Abstracts* **27;** 412.13

P.J. Brunton, C. Wilkinson & J.A. Russell (2001) Hypothalamo-Pituitary-Adrenal (HPA) Responses to Centrally Administered Orexin-A are Attenuated in Pregnant Rats. E*ndocrine Abstracts* **2**; OC10

Abstracts Presented at Conferences

P.J. Brunton, P.M. Bull and J.A. Russell (1999) Resetting Osmoregulation by Centrally Acting Relaxin and Sex Steroids. *Presented at the Maternal Brain conference, Bristol, UK.*

P.J. Brunton, P.M. Bull and J.A. Russell (1999) Relaxin and Reduced Osmoresponsiveness of Oxytocin Neurones in Pregnant Rats. *Presented at the British Neuroendocrine Group meeting, London, UK.*

P.J. Brunton, P.M. Bull and J.A. Russell (1999) The Effect of Relaxin on Neurohypophysial Oxytocin Secretion Following Osmotic Challenge. *Presented at the 1999 World Congress on Neurohypophysial Hormones, Edinburgh, UK.*

P.J.Brunton, A.Wigger, I.D.Neumann, A.J.Douglas & J.A.Russell (2000) Attenuated Stress Responses In Pregnant Rats: A Result of Altered Feed- Forward and Feedback Mechanisms? *Presented at the British Neuroendocrine Group Meeting, Bristol, UK.*

Brunton P.J., Ma S., Shipston M.J., Douglas A.J. and Russell J.A. (2000) Reduced Neuroendocrine Stress Responses To Immune Signals In Pregnant Rats. *Presented at the 3rd World Congress on Stress, Dublin, Ireland.*